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(54) Title: HYPOXIA-INDUCIBLE FACTOR 1 α HIF-1 α VARIANTS AND METHODS FOR IDENTIFYING HIF-1 α MODULATORS		
(57) Abstract The present invention relates to a method for identifying compounds capable of modulating the function of a functional domain of human HIF-1 α , said method comprising (i) contacting a candidate compound with a variant of human HIF-1 α , said variant essentially lacking at least one functional domain of human HIF-1 α , or having a mutation making at least one functional domain of human HIF-1 α essentially inactive, said functional domain or domains being selected from the group consisting of (a) the PAS-B domain located in human HIF-1 α between amino acids 178 and 390, (b) the C-terminal nuclear localization sequence (NLS) located in human HIF-1 α essentially at amino acids 718 to 721, and (c) the transactivator / coactivator domain (N-TAD) located in human HIF-1 α essentially between amino acids 531 to 584, and (d) the transactivator / coactivator domain (C-TAD) located in human HIF-1 α essentially between amino acids 813 and 826, and (ii) determining the effect of the candidate compound on the said variant.		

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HYPOXIA-INDUCIBLE FACTOR 1 α HIF-1 α VARIANTS AND METHOD FOR IDENTIFYING HIF-1 α MODULATORS

TECHNICAL FIELD

5 The present invention relates to novel variants of human hypoxia-inducible factor (HIF)-1 α , as well as to the use of the said variants in screening assays for the elucidation of functional motifs in HIF-1 α and for the identification of compounds that modulate the function of HIF-1 α , said compounds being potentially useful in the regulation of target genes normally associated with HIF-1 α such as genes involved in angiogenesis,
10 erythropoiesis, and glycolysis.

BACKGROUND ART

15 Oxygen plays a critical biological role as the terminal electron acceptor in the mitochondria of vertebrate cells. During evolution, these cells have developed ways to sense alterations in oxygen levels and, during this process, acquired the ability to conditionally modulate the expression of genes involved in adaptive physiological responses to hypoxia including angiogenesis, erythropoiesis, and glycolysis. These genes include vascular endothelial growth
20 factor, erythropoietin, several glycolytic enzymes and inducible nitric oxide synthase, and have all been shown to contain hypoxia responsive elements (HREs) (for reviews, see Guillemin and Krasnow (1997) Cell 89, 9-12; Wenger and Gassmann (1997) Biol. Chem. 378, 609-616). Under hypoxic conditions these response elements are recognized by a heterodimeric complex consisting of the hypoxia inducible factor (HIF)-1 α and Arnt (Wang
25 et al. (1995) Proc. Natl. Acad. Sci. USA 92, 5510-5514; Gradin et al. (1996) Mol. Cell. Biol. 16, 5221-5231). Both these transcription factors belong to the rapidly growing family of basic-helix-loop-helix (bHLH)-PAS (Per, Arnt, Sim) proteins. bHLH/PAS transcription factors play diverse biological roles.

30 The recent generation of HIF-1 α - and Arnt-deficient embryonal stem cells and mice have indicated critical roles of both these factors in cardiovascular development and regulation of

HRE-driven target genes (Maltepe et al. (1997) Nature 386, 403-407). The mechanism of hypoxia-dependent formation and activation of the HIF-1 α -Arnt complex is presently poorly understood. The present inventors and others have recently demonstrated that HIF-1 α protein levels are specifically and massively upregulated under hypoxic conditions in most if not in all cells. Since HIF-1 α mRNA levels are unaltered in response to hypoxia, this mode of regulation appears to occur via a post-transcriptional step involving stabilization of HIF-1 α protein levels (Huang et al. (1996) J. Biol. Chem. 271, 32253-32259; Kallio et al. (1997) Proc. Natl. Acad. Sci. USA 94, 5667-5672; Salceda and Caro (1997) J. Biol. Chem. 272, 22642-22647) preceding recruitment of Arnt and generation of a nuclear DNA binding complex.

Import of transcription factors into the nucleus is frequently a conditionally regulated process that occurs in response to various external and internal stimuli as well as to developmental cues (Vandromme et al. (1996) Trends Biochem. Sci. 21, 59-64). Thus, this process can constitute a critical mechanism of regulation of transcription factor activity. Active, energy-dependent transport of proteins to the nucleus requires the presence of one or several nuclear localization signals (NLSs) within the transported protein (or its interaction partner). NLS motifs are short amino acid moieties that can be divided into two main groups: (i) the SV40 large T antigen type of NLS, characterized by a single cluster of four or more consecutive basic residues; and (ii) a bipartite NLS, consisting of two basic residues, a spacer of any ten amino acids, and a basic cluster where three of the next five residues are basic (Vandromme et al., *supra*, and references therein).

Purified HIF-1 α , its amino acid sequence and polynucleotide sequence are disclosed by Wang et al. (1995) Proc. Natl. Acad. Sci. USA 92, 5510-5514 and in WO 96/39426. It has been suggested (Jiang et al. (1997) J. Biol. Chem. 272, 19253-19260) that amino acids 531-826 of HIF-1 α contain two transactivation domains, TAD-N (amino acids 531-575) and TAD-C (amino acids 786-826). Further, Pugh et al. ((1997) J. Biol. Chem. 272, 11205-11214) defined two minimal domains within HIF-1 α (amino acids 549-582 and 775-826) each of which could act to confer transcriptional activation.

There is a need for novel variants of human HIF-1 α , which variants can be used in screening assays for the elucidation of functional motifs in HIF-1 α and for the identification of compounds that modulate the function of HIF-1 α , said compounds being potentially useful in the regulation of target genes normally associated with HIF-1 α such as genes involved in angiogenesis, erythropoiesis, and glycolysis.

DESCRIPTION OF THE DRAWINGS

Fig. 1

HIF-1 α shows hypoxia-inducible nuclear translocation. COS7 cells were transiently transfected with pCMV-HIF-1 α and after 24 h expression incubated for 6 additional h under either normoxic (21% O₂) or hypoxic (1% O₂) conditions before fixation for immunocytochemistry. Localization of expressed HIF-1 α was determined by indirect immunofluorescence using anti-HIF-1 α antiserum as described in Materials and Methods.

Fig. 2

Hypoxic regulation of nuclear translocation by GFP-HIF-1 α . (A) Schematic representation showing the GFP and GFP-HIF-1 α protein constructs. For HIF-1 α , location of the basic helix-loop-helix and PAS (Per/Arnt/Sim) domains is also indicated. (B) Hypoxia and hypoxia-mimicking chemicals induce rapid nuclear translocation of GFP-HIF-1 α fusion proteins. COS7 cells were transiently transfected with either GFP or of GFP-HIF-1 α expression vectors and after 24 h expression induced for 6 h either with 1 % O₂, 100 μ M CoCl₂ or with 100 μ M 2,2'-dipyridyl (DP) before microscopy. (C) Endogenous HIF-1 α , but not GFP-HIF-1 α , protein levels are upregulated by hypoxic induction. COS7 cells were transfected with nonfusion GFP expression vector (lanes 1 and 2) or with GFP-HIF-1 α (lanes 3 and 4). Following transfection cells were exposed to 21% or 1% (hypoxia) oxygen for 30 h as indicated by - and + signs. Whole cell extracts were prepared as described in Materials and Methods, and 50 μ g aliquots were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using anti-HIF-1 α antiserum. GFP-HIF-1 α protein is indicated by an arrowhead, whereas the asterisk denotes endogenous HIF-1 α immunoreactivity. The

positions of the molecular weight markers (in kDa) are indicated on the left. (D) Time-dependent nuclear translocation of GFP-HIF-1 α . COS7 cells were transfected as in panel A with GFP-HIF-1 α expression vectors and induced with 100 μ M 2,2'-dipyridyl. Photographs were taken at the indicated timepoints after induction. (E) Graph presentation of the nuclear entry of GFP-HIF-1 α . Cells transfected with GFP-HIF-1 α were induced with 100 μ M 2,2'-dipyridyl and the a total of 250 cells were analyzed for distribution of the fluorescence at fixed timepoints. N (%) is the percentage of cells showing exclusively nuclear fluorescence.

Fig. 3

10 Nuclear export of HIF-1 α upon withdrawal of hypoxic signal. (A) Outline of the experimental strategy. COS7 cells were transfected with the GFP-HIF-1 α expression vector (6 μ g/60-mm dish) for 6 h and grown under atmospheric oxygen for 12, whereafter they were either induced with 100 μ M 2,2'-dipyridyl or left uninduced for 1 h. Subsequently 2,2'-dipyridyl was withdrawn by change of medium and washing of the cells, and the cells were
15 thereafter incubated in the absence (–) or presence (+) of 25 μ M cycloheximide for 24 h. During this time period cells were observed microscopically at different timepoints as indicated by arrows. For each timepoint, a total of 200 cells was counted and analyzed. (B) HIF-1 α is redistributed from the nucleus upon withdrawal of 2,2'-dipyridyl. The subcellular distribution of GFP-HIF-1 α is shown at different timepoints following withdrawal. The
20 percentage of cells belonging to each category is indicated (for classification, see Table I). For reference the intracellular distribution of GFP-HIF-1 α prior to induction with 2,2'-dipyridyl is also shown.

Fig. 4

25 Subcellular distribution of GFP-HIF-1 α fusion proteins. (A) Schematic presentation of fusion proteins. All expression constructs were assembled into pCMX-SAH/Y145F vector expressing a humanized GFP under the control of the CMV promoter. (B) Subcellular distribution of GFP-HIF-1 α chimeric proteins. Fusion constructs corresponding to those presented panel A were transfected into COS7 cells and after 24 h expression either 100 μ M
30 2,2'-dipyridyl (DP) or vehicle (H₂O) were added to the culture medium and incubated for 6 h before observation. Photographs were taken using a Zeiss fluorescent microscope.

Fig. 5

Identification of a hypoxia-inducible NLS motif within the C-terminus of HIF-1 α . (A) Analysis of human (h) and mouse (m) HIF-1 α sequences reveals two conserved nuclear localization signal (NLS) motifs. The N-terminal NLS motif is showing homology to the bipartite-like NLS sequence as exemplified by *X. laevis* nucleoplasmin, whereas the C-terminal NLS motif is related to SV40 large T antigen-like NLS sequence. (B) Effect of point mutagenesis within the NLS motifs on nuclear accumulation of GFP-HIF-1 α fusion proteins. COS7 cells were transfected with various GFP- HIF-1 α fusion protein expression plasmids carrying either wild-type or mutated HIF-1 α NLS sequences. The mutations were either single (Arg to Ala) or double (Arg to Gly) amino acid exchanges at residues 17 and 18, or a single amino acid exchange (Lys to Thr) at codon 719, respectively. After transfection, cells were treated with 100 μ M 2,2'-dipyridyl or with vehicle only for 6 h before fluorescence microscopy.

Fig. 6

Overlapping C-terminal structures mediate inducible nuclear translocation and transcriptional activation by HIF-1 α . (A) Transcriptional activation of a hypoxia responsive gene by GFP-HIF-1 α chimeras. COS7 cells were cotransfected either with GFP or GFP-HIF-1 α fusion constructs together with a hypoxia responsive reporter gene (HRE-luc). Six h after transfection cells were exposed either to 21% or 1% O₂ for 30 h before harvest. Normalized reporter gene activities are expressed relative to that of nonfusion GFP in normoxia. The results of two independent experiments performed in triplicates \pm SEM are shown. (B) Transcriptional activation by GFP-HIF-1 α is Arnt-dependent. COS7 cells were cotransfected either with GFP or GFP-HIF-1 α expression vectors and a hypoxia responsive reporter gene (HRE-luc in the presence of either wild-type Arnt or a dominant negative Arnt mutant, Arnt Δ b, lacking the DNA binding b domain. Cells were exposed for 21% or 1% O₂ for 30 h before harvest and reporter gene assays. Normalized reporter gene activities are expressed relative to that of nonfusion GFP in normoxia. The results of two independent experiments performed in triplicates \pm SEM are shown. (C) Functional analysis of GAL4-HIF-1 α fusion proteins. The same subregions of HIF-1 α as characterized in panel A were fused to the GAL4

DNA binding domain (GAL4 DBD) and transfected into COS7 cells together with a reporter plasmid expressing the luciferase gene driven by the thymidine kinase minimal promoter under the control of five copies of GAL4 binding sites. Cells were exposed for 21% or 1% O₂ for 30 h before harvest and reporter gene assays. Normalized reporter gene activities are expressed relative to that of the nonfusion GAL4 DBD in normoxia. The results of three independent experiments performed in triplicates +/- SEM are shown. (D) Constitutive nuclear localization of GAL4-HIF-1 α . COS7 cells were transiently transfected with pCMX-GAL4-HIF-1 α and after 24 h expression exposed to normoxic (21% O₂) or hypoxic (1% O₂) conditions for 6h before immunocytochemistry. Indirect immunofluorescence of expressed GAL4-HIF-1 α fusion protein was conducted with anti-GAL4-DBD antiserum, biotinylated anti-rabbit Ig antibody, and Texas Red conjugated with streptavidin.

Fig. 7

Hypoxia-dependent recruitment of the CBP coactivator by HIF-1 α . (A) Stimulation of hypoxia responsive gene expression by CBP. COS7 cells were cotransfected with either GFP-HIF-1 α or GFP-HIF-1 α K719T together with HRE-luciferase reporter and Rous sarcoma virus (RSV)-driven CBP expression vector (or empty expression plasmid). Six h after transfection cells were exposed either to 21% or 1% O₂ for 30 h before harvesting the cells. Luciferase values were normalized for transfection efficiency by cotransfection of alkaline phosphatase expressing pRSV-AF. The data are expressed relative to that of GFP-HIF-1 α in normoxia. (B) NLS mutation eliminating inducible nuclear import does not affect hypoxia dependent recruitment of CBP. GAL4-HIF-1 α or GAL4-HIF-1 α transactivation domain fusion proteins were transfected into COS7 cells together with a GAL4 responsive reporter plasmid with or without CBP expression plasmid. Cells were exposed for 21% or 1% O₂ for 30 h before harvest and reporter gene assays. (C) The C-terminal transactivation domain of HIF-1 α is targeted by CBP. GAL4 fusion protein containing the minimal C-terminal transactivation domain (amino acids 776-826) or a deletion mutant thereof were transfected into COS7 cells together with a GAL4 responsive reporter plasmid in the absence or presence of the CBP expression vector. Cells were exposed for 21% or 1% O₂ for 30 h before harvest and reporter gene assays. After normalization for transfection efficiency using alkaline

phosphatase activity, reporter gene activities are expressed relative to that of GAL4 in normoxia.

Fig. 8

5 The transcriptional coactivator TIF2 enhances the HIF-1 α -mediated transactivation function and interacts with HIF-1 α in vivo. COS7 cells were transiently cotransfected as described in Materials and Methods with 0.5 μ g pT81/HRE-Luc reporter construct, hHIF1- α expression vector (pCMV4/HIF-1 α , 0.2 μ g), Arnt expression vector (pCMV4/Arnt, 0.2 μ g) and 0.75-1.5 μ g of either TIF2 (pSG5/TIF2) or CBP (Rc/RSV-CBP-HA), as indicated. The total amount of
10 DNA was kept constant by the addition of parental pCMV4 when appropriate. Following transfection cells were incubated either under normoxic (21% O₂) or hypoxic conditions (1% O₂). Luciferase activities were normalized for transfection efficiency by cotransfection of alkaline phosphatase expressing pRSV-AF. Data are presented as luciferase activity relative to cells transfected with pCMV4 and reporter gene only and incubated at normoxia. Values
15 represent the mean \pm S.E. of two independent experiments.

Fig. 9

The transcriptional coactivator SRC-1 stimulates HIF-1 α activity in a hypoxia-dependent manner and interacts in vitro with HIF-1 α . (A) COS7 and (B) human embryonic kidney 293
20 cells were cotransfected with pT81/HRE-Luc (0.5 μ g), 0.2 μ g of pCMV4/HIF-1 α (hHIF1- α), 0.2 μ g of pCMV4/Arnt (Arnt), and 0.75-1.5 μ g of SRC-1 (pSG5/SRC-1), as indicated. Six h after transfection cells were exposed to either 21 or 1% O₂ for 36 h before harvest. Luciferase values are presented as relative luciferase activity as described in the legend to Fig. 1. The results of three independent experiments performed in duplicate \pm S.E. are shown. (C) SRC-1
25 PAS domain is not required for functional interaction with HIF-1 α . (Top) Schematic representation of full-length SRC-1 and SRC-1 Δ PAS. (Bottom) pGAL4/HIF 71-826 was cotransfected into COS7 cells together with a reporter plasmid expressing the luciferase gene driven by the thymidine kinase minimal promoter under the control of five copies of GAL4-binding sites, and 1.5 μ g of either full-length SRC-1 or a deletion mutant of SRC-1, SRC-
30 1 Δ PAS, lacking the PAS domain. Cells were exposed to 21 or 1% O₂ for 36 h before harvest and reporter gene assays. Luciferase values are presented as relative luciferase activity as

described in the legend to Fig. 1. The results of two independent experiments performed in duplicate \pm S.E. are shown. (D) In vitro interaction between SRC-1 and HIF-1 α . COS7 cells were transfected with 10 μ g of the expression plasmid pGST-HIF-1 α (GST-HIF) or empty GST expression vector (GST). Cells were exposed to either 100 μ M CoCl₂ or vehicle (H₂O) for 24 h. Cell extracts were prepared and incubated with [³⁵S]methionine-labeled in vitro translated SRC-1 protein. The complexes were immobilized on glutathione-agarose beads for 2 h and eluted with the sample buffer by boiling. The eluted material was analyzed on 5% SDS-PAGE and visualized by fluorography. *1/5 x Input* represents one-fifth of the amount of [³⁵S]methionine-labeled SRC-1 used in the binding reactions. Positions of molecular mass markers are shown on the left.

Fig. 10

LXXLL motifs of SRC-1 are not required for HIF-1 α -mediated transactivation function and interaction with HIF-1 α in vivo. Schematic representation of full-length SRC-1 and mutant SRC-1 M1234. Black bars represent the approximate locations of the LXXLL motifs in the linear SRC-1 sequence; circles with numbers indicate sites of mutation of LXXLL motifs in which conserved leucine residues are replaced by alanines. (Bottom) pGAL4/HIF 71-826 was cotransfected into COS7 cells together with a GAL4-responsive reporter plasmid, and 1.5 μ g of either full-length SRC-1 or mutated SRC-1, SRC-1 M1234. Cells were exposed to 21 or 1% O₂ for 36 h before harvest and reporter gene assays. Luciferase values are presented as relative luciferase activity as described in the legend to Fig. 1. The results of two independent experiments performed in duplicate \pm S.E. are shown.

Fig. 11

Definition of HIF-1 α structures which are regulated by the CBP and SRC-1 coactivators. (A) Different subregions of HIF-1 α were fused to the GAL4 DBD and transfected into COS7 cells together with a GAL4-responsive reporter plasmid in the absence or presence of 1.5 μ g of either CBP or SRC-1 expression plasmids. Cells were exposed to 21 or 1% O₂ for 36 h before harvest. (B) The C-terminal transactivation domain of Arnt mediates transcriptional activation by CBP and SRC-1. COS7 cells were cotransfected with the indicated GAL4/Arnt fusion proteins and 1.5 μ g of CBP or SRC-1 together with a GAL4-responsive reporter

plasmid. Six h after transfection cells were exposed to either 21 or 1% O₂ for 36 h before harvest and reporter gene assays. (C) HIF-1 α prevents Arnt from functionally interacting with CBP and SRC-1. GAL4/Arnt 128-774 and a deletion mutant (GAL4/Arnt 128-603) were transfected into COS7 cells together with 0.2 μ g hHIF-1 α expression vector and 1.5 μ g of CBP and SRC-1, as indicated. Luciferase activity was measured following 36 h of exposure to either 21 or 1% O₂. Normalized reporter gene activities are expressed relative to that of non-fusion GAL4 in normoxia. The results of three independent experiments performed in duplicate \pm S.E. are shown.

Fig. 12

CBP and SRC-1 cooperatively enhance HIF-1 α -mediated transcriptional activation. (A) COS7 cells were transiently cotransfected with pCMV4/HIF-1 α and 0.75-1.5 μ g of SRC-1 and/or 0.75-1.5 μ g of CBP expression plasmids together with a hypoxia-responsive reporter gene (pT81/HRE-Luc) and subsequently exposed to 21 or 1% O₂. Luciferase values are presented as relative luciferase activity as described in the legend to Fig. 1. The results of three independent experiments performed in duplicate \pm S.E. are shown. (B) Cooperative activation by CBP and SRC-1 of GAL4/HIF-1 α fusion proteins. COS7 cells were cotransfected with GAL4/HIF-1 α fusion constructs together with a GAL4-responsive reporter plasmid in the absence or presence of CBP and/or SRC-1, as indicated (in μ g). Cells were exposed to 21 or 1% O₂ before harvest. After normalization for transfection efficiency using alkaline phosphatase activity, reporter gene activities are expressed as relative to that of GAL4 in normoxia. The results of two independent experiments performed in duplicate \pm S.E. are shown. (C) p300 protein stimulates the activity of the two minimal transactivation domains of HIF-1 α in a hypoxia-dependent manner. (Right Panel) Schematic representation of full-length p300 (pCMV β -p300-HA) and p300 Δ (pCMV β -p300 Δ 1254-1376) (Left Panel) The two minimal transactivation domains of HIF-1 α were fused to GAL4 DBD and transfected into COS7 cells together with a GAL4-responsive reporter plasmid in the absence or presence of p300, p300 Δ and/or SRC-1 expression vectors, as indicated. Cells were exposed to 21 or 1% O₂ before harvest. Normalized reporter gene activities are expressed as relative to that of GAL4 in normoxia. The results of a representative experiment performed in duplicate are shown.

Fig. 13

Ref-1 enhances HIF-1 α function. (A) COS7 cells were cotransfected with different GAL4/HIF-1 α fusion constructs together with a GAL4-responsive reporter plasmid in the absence or presence of 1.5 μ g of Ref-1 (pCMV5/Ref-1), as indicated. Cells were exposed to 21 or 1% O₂ before harvest. After normalization for transfection efficiency using alkaline phosphatase activity, reporter gene activities are expressed as relative to that of GAL4 in normoxia. The results of two independent experiments performed in duplicate \pm S.E. are shown. (B) Ref-1 potentiates CBP and SRC-1 activation of HIF-1 α . The same GAL4/HIF-1 α fusion proteins as shown in (A) were cotransfected into COS7 cells together with a GAL4-responsive reporter plasmid in the absence or presence of different combinations of Ref-1 (0.75 μ g), CBP (0.75 μ g), and/or SRC-1 (0.75 μ g), as indicated. The bottom panel shows an enlargement of the area marked with dots.

Fig. 14

Model of multiple levels of regulation of HIF-1 α activity. Hypoxia leads to inhibition of proteasome-mediated degradation and consequently to stabilization of the protein (1), and stimulates the nuclear import of HIF-1 α by unmasking its nuclear localization signal (2). Subsequently, the Arnt partner factor is recruited HIF-1 α in the nucleus and the resulting HIF-1 α /Arnt heterodimer recognizes hypoxia responsive elements (HREs) of target genes and (3) functionally interacts with transcriptional coactivators (exemplified by CBP/p300) to activate target promoters. The order of events within the nucleus (Arnt dimerization, DNA binding, and recruitment of CBP) is not yet known.

DISCLOSURE OF THE INVENTION

It has surprisingly been shown that the mechanism of activation of HIF-1 α is a multi-step process, which includes hypoxia-dependent nuclear import and activation (derepression) of the transactivation domain, resulting in recruitment of the CBP/p300 coactivator. Inducible nuclear accumulation has been shown to be dependent on a nuclear localization signal (NLS)

within the C-terminal end of HIF-1 α that also harbors the hypoxia-inducible transactivation domain. Nuclear import of HIF-1 α was inhibited by either deletion or a single amino acid substitution within the NLS sequence motif and, within the context of the full-length protein, these mutations also resulted in inhibition of the transactivation activity of HIF-1 α and recruitment of CBP. It has further been shown that nuclear localization *per se* is not sufficient for transcriptional activation, since fusion of HIF-1 α to the heterologous GAL4 DNA binding domain generated a protein which showed constitutive nuclear localization but required hypoxic stimuli for function as a CBP-dependent transcription factor.

Moreover, the transcriptional coactivators SRC-1 and TIF2 also support HIF-1 α -dependent transcriptional activation. CBP and the SRC-1/TIF2 class of coactivators target both N- and C-terminal transactivation domains of HIF-1 α , and are potentiated in their function by the redox regulatory enzyme Ref-1. In fact, Ref-1 physically interacts with HIF-1 α under hypoxic conditions. Thus, hypoxia-inducible nuclear import and transactivation by Ref-1-dependent recruitment of the transcriptional coactivators CBP, SRC-1/TIF2 can be functionally separated from one another and play critical roles in signal transduction by HIF-1 α .

Consequently, in a first aspect this invention relates to a method for identifying compounds capable of modulating the function of a functional domain of human HIF-1 α , said method comprising

(i) contacting a candidate compound with a variant of human HIF-1 α , said variant essentially lacking at least one functional domain of human HIF-1 α , or having a mutation making at least one functional domain of human HIF-1 α essentially inactive, said functional domain or domains being selected from the group consisting of

- (a) the PAS-B domain located in human HIF-1 α between amino acids 178 and 390,
- (b) the C-terminal nuclear localization sequence (NLS) located in human HIF-1 α essentially at amino acids 718 to 721, and
- (c) the transactivator domain (N-TAD) located in human HIF-1 α essentially between amino acids 531 to 584, and
- (d) the transactivator domain (C-TAD) located in human HIF-1 α essentially between amino acids 813 and 826, and

(ii) determining the effect of the candidate compound on the said variant.

In one preferred form, the said method comprises (i) contacting a candidate compound with a cell expressing a said variant of human HIF-1 α , said variant being conjugated to a molecular probe, and (ii) detecting the localization of the said molecular probe within the cell. The said molecular probe is preferably a fluorescent probe, such as the *Aequorea victoria* Green Fluorescent Protein, or a variant thereof. A change in the relative fluorescence of the nucleus to the cytoplasm is then indicative for a compound capable of modulating the function of the said functional domain.

In another preferred form of the invention, the said variant of human HIF-1 α is regulating the activity of a reporter gene; a change in the activity of the reporter gene being indicative of a compound capable of modulating the function of the said functional domain. The HIF-1 α variant can e.g. be fused to the DNA binding domain of GAL4, and the cells expressing the HIF-1 α variant will contain a GAL4 response element operatively linked to a reporter gene. Alternatively, the variant of human HIF-1 α can be fused to the *Aequorea victoria* Green Fluorescent Protein, and the cells expressing the HIF-1 α variant could suitably contain an erythropoietin hypoxia response element operatively linked to a reporter gene, e.g. a firefly luciferase gene.

The method according to the invention can suitably be carried out in the presence of a transcriptional coactivator, in particular one or more of the transcriptional coactivators designated SRC-1, TIF2 or CBP. When a transcriptional coactivator is present, the method according to the invention can suitably be carried out in the further presence of the redox regulatory protein designated Ref-1.

When the said functional domain of human HIF-1 α is the PAS-B domain, the said variant of human HIF-1 α can e.g. be HIF-1 α /1-74 (SEQ ID NO: 2); HIF-1 α /1-245 (SEQ ID NO: 3); HIF-1 α /Δ178-390 (SEQ ID NO: 6); HIF-1 α /331-641 (SEQ ID NO: 7); HIF-1 α /526-641 (SEQ ID NO: 8); HIF-1 α /526-813 (SEQ ID NO: 9); or HIF-1 α /526-826 (SEQ ID NO: 10).

When the said functional domain of human HIF-1 α is the C-terminal nuclear localization sequence (NLS) domain, the said variant of human HIF-1 α can e.g. be HIF-1 α /1-74 (SEQ ID NO: 2); HIF-1 α /1-245 (SEQ ID NO: 3); HIF-1 α /1-330 (SEQ ID NO: 4); HIF-1 α /1-652 (SEQ ID NO: 5); HIF-1 α /331-641 (SEQ ID NO: 7); or HIF-1 α /526-641 (SEQ ID NO: 8).

When the said functional domain of human HIF-1 α is the transactivator domain (N-TAD), located in human HIF-1 α essentially between amino acids 531 to 584, the said variant of human HIF-1 α can e.g. be HIF-1 α /1-74 (SEQ ID NO: 2); HIF-1 α /1-245 (SEQ ID NO: 3); HIF-1 α /1-330 (SEQ ID NO: 4); HIF-1 α /776-826 (SEQ ID NO: 14); or HIF-1 α /776-813 (SEQ ID NO: 15).

When the said functional domain of human HIF-1 α is the transactivator domain (C-TAD), located in human HIF-1 α essentially between amino acids 813 and 826, the said variant of human HIF-1 α can e.g. be HIF-1 α /1-74 (SEQ ID NO: 2); HIF-1 α /1-245 (SEQ ID NO: 3); HIF-1 α /1-330 (SEQ ID NO: 4); HIF-1 α /1-652 (SEQ ID NO: 5); HIF-1 α /331-641 (SEQ ID NO: 7); HIF-1 α /526-641 (SEQ ID NO: 8); HIF-1 α /526-813 (SEQ ID NO: 9); HIF-1 α /1-813 (SEQ ID NO: 12); HIF-1 α /531-584 (SEQ ID NO: 13); HIF-1 α /776-813 (SEQ ID NO: 15).

In a further aspect, the invention provides an isolated variant of human HIF-1 α , said variant being selected from the group consisting of HIF-1 α /1-74 (SEQ ID NO: 2); HIF-1 α /1-245 (SEQ ID NO: 3); HIF-1 α /1-330 (SEQ ID NO: 4); HIF-1 α /1-652 (SEQ ID NO: 5); HIF-1 α / Δ 178-390 (SEQ ID NO: 6); HIF-1 α /331-641 (SEQ ID NO: 7); HIF-1 α /526-641 (SEQ ID NO: 8); HIF-1 α /526-813 (SEQ ID NO: 9); HIF-1 α /526-826 (SEQ ID NO: 10); HIF-1 α /71-826 (SEQ ID NO: 11); HIF-1 α /1-813 (SEQ ID NO: 12); HIF-1 α /531-584 (SEQ ID NO: 13); HIF-1 α /776-826 (SEQ ID NO: 14); and HIF-1 α /776-813 (SEQ ID NO: 15).

EXAMPLES

1. HIF-1 α shows hypoxia-inducible nuclear import

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To study the intracellular localization of HIF-1 α we transiently transfected simian COS7 cells with a CMV promoter-driven HIF-1 α expression vector. Following 24 h of expression cells were incubated for a further 6 h under either normoxic (21% O₂) or hypoxic (1% O₂) conditions. Immunostaining with polyclonal anti-HIF-1 α antibodies (Kallio *et al.*, 1997) showed HIF-1 α immunoreactivity predominantly localized in the cytoplasm of the normoxic cells with some reactivity also detected in the nuclear compartment. Interestingly, exposure of the cells to hypoxia resulted in a very striking nuclear accumulation of HIF-1 α with hardly any detectable immunoreactivity remaining in the cytoplasm (Figure 1).

15 To further investigate the mechanism of activation of HIF-1 α and its subcellular localization in living cells we constructed a vector carrying an in-frame GFP-fusion of full-length HIF-1 α (schematically shown in Figure 2A) expressed under the control of the CMV promoter. Following transient transfection of COS7 cells with either the parental GFP construct or the chimeric GFP-HIF-1 α construct, fluorescence was observed in about 20-30% of the cells, reflecting the level of transfection efficiency. The fluorescence of GFP alone was uniformly localized throughout the cell, and hypoxic treatment had no effect either on the intensity or the subcellular distribution of fluorescence activity (Figure 2B). Transient expression of the GFP-HIF-1 α construct in normoxic cells resulted in signal mimicking the picture observed with GFP alone, thus showing fluorescence activity throughout the cell (Figure 2B).

25

For quantitative purposes, 200-300 fluorescent cells were routinely analyzed for compartmentalization of HIF-1 α and subdivided into four categories (Ylikomi *et al.* (1992) EMBO J. 11, 3681-3694): N, cells containing exclusively nuclear fluorescence; N > C, cells in which the nuclear fluorescence dominates over cytoplasmic fluorescence; N = C, cells having equal distribution of fluorescence in the cytoplasmic and nuclear compartments; and N < C, cells in which the intensity of fluorescence in the cytoplasm exceeds that in the

30

nucleus. A percentage of cells belonging to each category is indicated in Table I. Under normoxic conditions 65% of cells transiently expressing the GFP-HIF-1 α construct belonged to category N = C; whereas a limited number the cells (8%) showed preferential nuclear compartmentalization of HIF-1 α (category N). In contrast, exposure of the cells to hypoxia (1% O₂) resulted in almost complete nuclear accumulation of GFP-HIF-1 α (Figure 2B), where 90% of the transfected cells exhibited exclusively nuclear fluorescence (category N; Table I). Moreover, treatment of the cells with chemicals known to mimic hypoxic induction of target gene expression (Wenger and Gassmann (1997) Biol. Chem. 378, 609-616) and induction of HIF-1 α DNA binding activity (Wang et al. (1995) Proc. Natl. Acad. Sci. USA 92, 5510-5514; Gradin et al. (1996) Mol. Cell Biol. 16, 5221-5231) e.g. CoCl₂ or the iron chelator 2,2'-dipyridyl (DP), also induced nuclear import of GFP-HIF-1 α . In fact, we did not observe any quantitative or qualitative differences in the potencies of any of these treatments to induce nuclear translocation of HIF-1 α (Figure 2B).

We and others have recently demonstrated that a critical mechanism in activation of HIF-1 α in response to hypoxia is upregulation of HIF-1 α protein levels. This response depends upon the stabilization of HIF-1 α protein rather than being the result of increased HIF-1 α mRNA expression levels or enhanced translation of HIF-1 α mRNA (Huang et al. (1996) J. Biol. Chem. 271, 32253-32259; Kallio et al. (1997) Proc. Natl. Acad. Sci. USA 94, 5667-5672; Salceda and Caro (1997) J. Biol. Chem. 272, 22642-22647). Since we did not detect any significant alterations in the relative intensity in fluorescence by GFP-HIF-1 α upon hypoxic stimulation (Figure 2B), we examined by immunoblot analysis the expression levels of GFP-HIF-1 α protein in transiently transfected normoxic cells or cells exposed to hypoxia (1% oxygen) following transfection. As shown in Figure 2C, no increase was detected in the expression levels of GFP-HIF-1 α protein in hypoxic cells as compared to normoxic ones (compare lanes 3 and 4). As a reference, however, endogenous HIF-1 α levels were upregulated in extracts from cells exposed to hypoxia (Figure 2C, lanes 2 and 4, endogenous HIF-1 α indicated by a star). In fact, as documented earlier (Kallio et al., *supra*), due to rapid turn-over endogenous HIF-1 α is virtually undetectable in extracts from normoxic cells (Figure 2C, lanes 1 and 3). These experiments therefore demonstrate that GFP efficiently stabilized HIF-1 α in normoxic cells, and that the use of GFP fusion proteins enabled us to

bypass the early level in regulation of HIF-1 α function, and allowed us to study intracellular compartmentalization of HIF-1 α without the added complexity of regulation of protein turnover.

5 Upon exposure of COS7 cells to hypoxia or hypoxia-mimicking chemicals such as 2,2'-dipyridyl the kinetics of nuclear accumulation of GFP-HIF-1 α showed half-maximal time of nuclear transfer of about 30 min (Figures 2D and E) and a complete nuclear import of the protein following 1 h of exposure at +37°C. These kinetics are quite slow in comparison to other transcription factors which show signal-inducible nuclear import. For instance, a
10 significantly more rapid time course of inducible nuclear transport has been reported for the glucocorticoid receptor (Ogawa et al. (1995) Proc. Natl. Acad. Sci. USA 92, 11899-11903; Carey et al. (1996) J. Cell Biol. 133, 985-996; Htun et al. (1996) Proc. Natl. Acad. Sci. USA 93, 4845-4850), a ligand-dependent transcription factor showing hormone-dependent nuclear translocation.

15

To further characterize hypoxic signal-dependent regulation of compartmentalization of HIF-1 α in living cells we next asked whether return of the cells to normoxia following exposure to hypoxia or withdrawal of hypoxia-mimicking chemicals would affect the intracellular localization of GFP-HIF-1 α . To this end we first induced nuclear import of GFP-HIF-1 α
20 (84% of cells belonging to category N) by treatment of transfected cells with 2,2'-dipyridyl for 1 h. Subsequently 2,2'-dipyridyl was withdrawn by washing of the cells in medium, and the cells were further incubated at normal levels of atmospheric oxygen tension (schematically represented in Figure 3A). After 12 h of incubation under normoxic conditions, 75% of the transfected cells showed an equal distribution of GFP-HIF-1 α in both
25 the nuclear and cytoplasmic compartments (category C = N), and the exclusively nuclear pool of GFP-HIF-1 α (category N) had been reduced from 84% to 5% (Figure 3B). An almost identical distribution of GFP-HIF-1 α was observed after 24 h of withdrawal of 2,2'-dipyridyl (Figure 3B). Thus, hypoxia-induced nuclear retention of GFP-HIF-1 α could be reversed following withdrawal of the hypoxic signal. To address the question whether this reversion
30 could depend on inefficient nuclear import of *de novo* synthesized GFP-HIF-1 α , we also performed this withdrawal experiment in the presence of the protein synthesis inhibitor

cycloheximide. Under these conditions, reversal of 2,2'-dipyridyl-induced nuclear retention of GFP-HIF-1 α was very similar to that observed in the absence of cycloheximide, i.e. reduction of the nuclear pool (category N) of GFP-HIF-1 α from 84% to 3%, concomitant with an increase of the pool of GFP-HIF-1 α belonging to category C=N from 6% to 82% (Figure 3B), indicative of an export of the originally nuclear pool of protein.

2. Role of the PAS domain in regulation of nuclear import of HIF-1 α

In order to identify the structural motif(s) involved in nuclear import of HIF-1 α , we generated a number of expression vectors containing GFP fused to different subdomains of HIF-1 α (schematically represented in Figure 4A). Following transient expression of these constructs in COS7 cells, the cells were either exposed to 2,2'-dipyridyl or left untreated for 6 h, and subsequently analyzed for compartmentalization of the fusion proteins. As shown in Figure 4B, fusion of the most N-terminal region of HIF-1 α spanning the bHLH motif (aa 1-74; SEQ ID NO: 2) to GFP resulted in a predominantly nuclear accumulation of the protein under normoxic conditions. Moreover, nuclear accumulation of this protein was not altered by exposure to 2,2'-dipyridyl (Figure 4B and Table I). It has previously been demonstrated that the basic DNA binding region within the bHLH domain is sufficient for nuclear localization of the bHLH-PAS transcription factors Arnt and the dioxin (aryl hydrocarbon) receptor (Eguchi et al. (1997) J. Biol. Chem. 272, 17640-17647; Ikuta et al. (1998) J. Biol. Chem. 273, 2895-2904). Thus, in analogy to these proteins, the isolated bHLH domain of HIF-1 α mediated constitutive nuclear import. In excellent agreement with these observations, HIF-1 α contains between aa 17-33 a bipartite NLS motif similar to that of *Xenopus laevis* nucleoplasmin (Dingwall et al. (1987) EMBO J. 6, 69-74) (Figure 5A).

Interestingly, extension of the N-terminal portion of HIF-1 α to fragments spanning, in addition to the bHLH domain, the PAS-A motif (HIF-1 α /1-245; Figure 4A; SEQ ID NO: 3) or the entire PAS domain (HIF-1 α /1-330; SEQ ID NO: 4) generated GFP-fusion proteins where the constitutive NLS motif within the bHLH domain appeared to be masked. Unlike HIF-1 α /1-74, these two proteins showed significant cytoplasmic fluorescence activity (Figure 4B) which was detectable both under normoxic and hypoxic conditions and generated a score

of 26 and 95% of the transfected cells within the category $N = C$, respectively (Table I). These results suggest that the PAS domain harbors a structure repressing nuclear import mediated by the NLS motif within the bHLH domain of HIF-1 α , resulting in cytoplasmic retention of the protein. In strong support of this of this model, GFP fusion protein containing a HIF-1 α mutant lacking a significant portion of the PAS domain, most notably the PAS B motif (HIF-1 α /Δ178-390; Figure 4A; SEQ ID NO: 6), fluorescence was predominantly nuclear already under normoxic conditions (Figure 4B), yielding a nuclear score (categories N and $N > C$) of 80% (Table I). Exposure to hypoxia had no further effect on the subcellular localization of GFP-HIF-1 α /Δ178-390 (Figure 4B; Table I). Thus, removal of the inhibitory motif or cytoplasmic retention signal within the PAS domain of HIF-1 α resulted in uncoupling of the protein from hypoxia regulation.

3. Identification of an NLS motif within the C-terminus of HIF-1 α

To further investigate which structural motifs mediated hypoxia-inducible nuclear compartmentalization of HIF-1 α and to map the functional architecture of HIF-1 α we next examined the intracellular localization of GFP fusion proteins containing HIF-1 α structures located C-terminally of the PAS domain, i.e. the fusion proteins GFP-HIF-1 α /331-641 and GFP-HIF-1 α /526-641 (Figure 4A) (The sequences of HIF-1 α /331-641 and HIF-1 α /526-641 are shown as SEQ ID NOS: 7 and 8, respectively). These two proteins demonstrated fluorescence both in the cytoplasm and nucleus (100% of the cells belonging to category $N = C$; Table I) that was unaltered by hypoxia and thus indistinguishable from the properties of the parental GFP protein (Figure 4B). Consistent with our observations above that the PAS domain represses nuclear import mediated by the constitutive NLS motif within the N-terminal bHLH domain, a fusion protein containing the bHLH/PAS domain and the C-terminal portion of HIF-1 α extending to aa 652 (GFP-HIF-1 α /1-652; The sequence of HIF-1 α /1-652 is shown as SEQ ID NO: 5) showed exclusively cytoplasmic/perinuclear localization (Figure 4B) with 100% of the transfected cells falling into category $N < C$ (Table I). Importantly, unlike GFP fused to full-length HIF-1 α , GFP-HIF-1 α /1-652 was non-responsive to hypoxia or dipyrindyl treatment and thus unable to undergo nuclear translocation under these conditions, indicating that the very C-terminus of HIF-1 α harbors sequences that

may function as important determinants for inducible nuclear accumulation. Strikingly, fusion to GFP of the C-terminal portion of HIF-1 α spanning amino acids 526-826 (SEQ ID NO: 10) generated a protein demonstrating exclusively nuclear fluorescence (Figure 4B). Thus, as shown schematically in Figure 5A, HIF-1 α contains a second NLS motif within this region of the protein. In fact, sequence analysis indicated the presence within this portion of HIF-1 α at amino acids 718 a bona fide SV40 Large T antigen type of NLS motif characterized by a single cluster of four consecutive basic residues, RKRK. This motif is also conserved in the mouse HIF-1 α protein (Figure 5A). Nuclear fluorescence by GFP-HIF-1 α /526-826 containing this motif was detected both under normoxic and hypoxic conditions, demonstrating that the domain of HIF-1 α harboring this second, SV40 Large T antigen type of NLS motif mediated constitutive nuclear import (Figure 4B; Table I).

4. A single amino acid mutation in the C-terminal NLS motif abolishes hypoxia-inducible nuclear import of full length HIF-1 α

Given the presence of a second NLS motif within the C terminus of HIF-1 α and the inability of the N-terminal portions of HIF-1 α to show hypoxia-inducible nuclear accumulation we investigated the role of the N- and C-terminal NLS motifs in inducible nuclear import by mutation analysis. To this end we introduced within the context of full length HIF-1 α either single or or double exchanges of the N-terminal Arg residues of the N-terminal bipartite NLS motifs of HIF-1 α , or mutated Lys 719 to Thr within the C-terminal NLS motif (Figure 5A). As shown in Figure 5B, point mutations of the N-terminal NLS motif did not affect hypoxia-inducible nuclear translocation of HIF-1 α . Importantly, however, the single amino acid exchange within the C-terminal NLS motif resulted in a dramatic reduction of the ability of the HIF-1 α to accumulate in the nucleus in response to hypoxia (Figure 5B) yielding a score of predominantly cytoplasmatically localized protein (category N < C) of 96% during normoxia versus 81% under hypoxic conditions (Table II). In conclusion, these experiments demonstrate that the C-terminal NLS motif of HIF-1 α mediates hypoxia-inducible nuclear import of the protein.

Introduction of the identical amino acid exchange within the C-terminal NLS motif severely impaired the constitutive nuclear accumulation of the isolated C-terminus of HIF-1 α fused to GFP (compare GFP-HIF-1 α /526-826 in Table I to GFP-HIF-1 α /526-826K719T in Table II). Thus, whereas the isolated C-terminal NLS motif mediates constitutive activity, it shows
5 hypoxia-inducible activity within the context of full length HIF-1 α . Since GFP-HIF-1 α /Δ178-390 lacking a major part of the PAS domain, most notably the PAS-B motif (Figure 4A), shows constitutive nuclear localization (Figures 4B and 5B), it appears that the C-terminal NLS motif functions in close partnership with the PAS domain in mediating the inducible nuclear import response. To test this notion, we also introduced the single amino acid
10 exchange of Lys 719 to Thr into the PAS B deletion mutant GFP-HIF-1 α /Δ178-390. Interestingly, this double mutant failed to show any significant nuclear import under either normoxic or hypoxic conditions (Figure 5B), generating scores of protein predominantly localized in the cytoplasmic compartment (category N < C) of 82 and 85%, respectively (Table II). In summary, these results demonstrate that the N-terminal basic region does not
15 function as an NLS motif in the context of the full-length protein and that the C-terminal NLS motif plays a critical in mediating inducible nuclear import of HIF-1 α .

5. Evidence for a multi-step activation pathway of HIF-1 α in response to hypoxia

20 We next examined the ability of GFP- HIF-1 α fusion proteins in functional cotransfection assays where GFP-HIF-1 α expression vectors were introduced into COS7 cells together with a hypoxia-responsive gene reporter construct carrying three tandem copies of the erythropoietin hypoxia responsive element (HRE) in front of the Herpes simplex thymidine kinase promoter and the luciferase gene. Reporter gene activity was not significantly altered
25 by hypoxic treatment (1% O₂; Figure 6A) or incubation with 2,2'-dipyridyl (data not shown) either in the absence of coexpressed proteins or in the presence of transiently expressed parental GFP, most probably due to low levels of endogenous HIF-1 activity in COS7 cells. However, coexpression of GFP-HIF-1 α resulted in stimulation (about 2-fold) of reporter gene activity already at normoxia, possibly due to stabilization of HIF-1 α levels by the GFP
30 moiety and, as described above, a small but detectable pool of HIF-1 α localized in the nucleus under these conditions. This reporter gene activity was further stimulated about 4-

fold by hypoxia (Figure 6A). In control experiments, transient expression of wild-type HIF-1 α also yielded a similar pattern of reporter gene activation, albeit with a slightly lower potency generating about a 2-fold activation response upon exposure to hypoxia. Thus, fusion of GFP to the immediate proximity of the N-terminal basic (DNA binding) region did not
5 interfere with transcriptional activation. In fact, stabilization of the fusion protein (Figure 2C) by the GFP moiety may rather have enhanced the potency of the activation response produced by HIF-1 α . As expected, in this functional assay GFP-HIF-1 α fusion proteins lacking the DNA binding bHLH region (GFP-HIF-1 α /331-641, GFP-HIF-1 α /526-641 and GFP-HIF-1 α /526-826) did not induce any reporter gene activity.

10 Although ectopic expression of wild-type Arnt did not significantly enhance hypoxia-induced transcriptional activation by GFP-HIF-1 α , transient expression of a dominant negative Arnt mutant, Arnt Δ b, lacking the DNA binding basic (b) domain (Lindebro et al. (1995) EMBO J. 14, 3528-3539), resulted in potent inhibition of the hypoxia-dependent activation response
15 (Figure 6B). Thus, these results demonstrate that hypoxia-induced transcriptional activation of HRE-driven reporter gene expression by GFP-HIF-1 α was critically dependent on interaction with the bHLH-PAS partner factor Arnt. In line with these results the mutant GFP-HIF-1 α / Δ 178-390 lacking the PAS-B domain, an important dimerization interface with Arnt (Lindebro et al., *supra*), failed to significantly induce HRE-dependent reporter gene
20 expression in response to hypoxia (Figure 6A).

To analyze functional activities of HIF-1 α independently of the endogenous DNA binding and dimerization domains and the Arnt partner, we next fused HIF-1 α to the heterologous DNA binding domain of GAL4. Functional activity of the fusion protein was monitored in
25 COS7 cells by a cotransfection assay using a reporter gene construct carrying five GAL4 DNA binding elements in front of thymidine kinase promoter and the luciferase gene. Six hours after transfection media was changed, and cells were either left uninduced or exposed to 1% oxygen for 30 h prior to harvest. In the presence of either empty expression vector or a vector expressing the minimal GAL4 DNA binding domain, reporter gene activity was
30 unaltered by reduced oxygen levels. Transient expression of GAL4-HIF-1 α at normoxia resulted in moderate (about 4- to 5-fold) reporter gene activation (Figure 6C). However,

when cells were incubated at 1% oxygen (Figure 6C; or with 100 μ M 2,2'-dipyridyl; data not shown), the functional activity of GAL4-HIF-1 α was further enhanced about 2-fold, resulting in about 8- to 10-fold stimulation of reporter gene activity over the background levels (Figure 6C). The GAL4 DNA binding domain is known to harbor an NLS motif capable of taking
5 fusion proteins to the nucleus (Ma and Ptashne (1987) Cell 51, 113-119). We therefore used antibodies directed against the DNA binding domain of GAL4 to examine in both normoxic and hypoxic cells the intracellular localization of the GAL4-HIF-1 α fusion protein by immunofluorescence. As shown in Figure 6D, the GAL 4 DNA binding domain mediated constitutive nuclear localization of the GAL4-HIF-1 α fusion protein (Figure 6D). Given the
10 fact that GAL4-HIF-1 α showed hypoxia-inducible functional activity these data establish that inducible nuclear import of HIF-1 α does not suffice for transcriptional activation, indicating a multi-step activation pathway HIF-1 α upon exposure to hypoxia.

6. Overlapping C-terminal structures mediate inducible nuclear translocation and 15 transcriptional activation by HIF-1 α

Based on deletion analysis HIF-1 α has been reported to harbor two transactivation domains (Jiang et al. (1997) J. Biol. Chem. 272, 19253-19260; Pugh et al. (1997) J. Biol. Chem. 272, 11205-11214). Whereas conflicting data exist with regard to hypoxia inducibility of the
20 expression levels of these two domains, the minimal transactivation domains have been mapped to amino acid residues 531-582 and 775-826. Consistent with these observations GFP-HIF-1 α fragments lacking transactivation domains but showing constitutive nuclear localization (e.g. GFP-HIF-1 α /1-74) did not induce reporter gene activity. In a similar fashion the fusion proteins GFP-HIF-1 α /1-245 and GFP-HIF-1 α /1-330 were devoid of any functional
25 activity (data not shown). Moreover, transient expression of GFP-HIF-1 α /1-652 failed to stimulate HRE-dependent reporter gene activity over background values (Figure 6A), although this protein contains one of the transactivation domains of HIF-1 α , resulting in transcriptional activation when fused to the GAL 4 DNA binding domain (Figure 6C), and was expressed at levels similar to those of transiently expressed GFP-HIF-1 α (data not
30 shown). This result is in excellent agreement with the inability of GFP-HIF-1 α /1-652 to enter the cell nucleus (Figure 4B). In conclusion, these data demonstrate that the inducible nuclear

localization and transactivation functions, although contained within the C-terminus of HIF-1 α , represent distinct functional entities, which can be separated from one another. This conclusion is further strengthened by the finding that deletion of the 13 most C-terminal amino acid residues from HIF-1 α resulted in significant reduction of transcriptional activity, both in the case of GFP-HIF-1 α /1-813 and GAL4-HIF-1 α /1-813 (Figures 6A and 6C), although both of these proteins were capable of entering the nucleus (Figure 4B and data not shown).

7. Transcriptional activation by HIF-1 α - hypoxia-dependent recruitment of the CBP coactivator is functionally uncoupled from the inducible NLS motif

The CREB binding protein CBP is a transcriptional coactivator protein known to interact with a number of constitutively active or inducible DNA binding transcription factors including, among others, CREB, c-Fos, c-Jun, various members of the steroid receptor superfamily, and p53 (Shikama et al. (1997) Trends Cell Biol. 7, 230-236). CBP has also been demonstrated to physically associate with HIF-1 α and to play a role in hypoxia-dependent transactivation of erythropoietin promoter in Hep3B cells (Arany et al. (1996) Proc. Natl. Acad. Sci. USA 93, 12969-12973). We therefore initially examined the effect of overexpression of CBP on hypoxia-dependent activation by GFP-HIF-1 α of the minimal HRE-driven reporter gene. As expected, transient expression of GFP-HIF-1 α under hypoxic conditions resulted in about 4- to 5-fold stimulation of the HRE-dependent reporter gene as compared to the activation response observed under normoxic conditions (Figures 6A and 7A). Coexpression of GFP-HIF-1 α with CBP further enhanced the hypoxia-dependent activation response by about 3-fold, resulting in about 15-fold stimulation of reporter gene activity over the levels observed at normoxia, further establishing that CBP supports transcription by the HIF-1 α /Arnt complex (Figure 7A). In excellent agreement with the data demonstrating that the single-amino acid mutant GFP-HIF-1 α K719T protein was unable to undergo inducible nuclear accumulation (Figure 5B and Table II), GFP-HIF-1 α K719T failed to produce the hypoxia-dependent activation response. Moreover, coexpression of GFP-HIF-1 α K719T with CBP did not produce any significant stimulation of reporter gene activity under hypoxic conditions, indicating the inability of the nuclear translocation-deficient HIF-

1 α protein to functionally interact with CBP. As a further control, we examined whether exposure to hypoxia affected the function of other transcription factors that are targets for regulation by CBP. In these experiments, we observed in reporter gene assays no effect of hypoxia on ligand-dependent enhancement by CBP of the functional activity of the retinoic acid receptor α (data not shown), a well-characterized target of CBP (for a review, see Torchia et al. (1998) *Curr. Opin. Cell Biol.* 10, 373-383).

As outlined above, fusion of a HIF-1 α to the GAL4 DNA binding domain generated a chimeric protein that produced modest (about 2-fold) hypoxia-dependent activation of reporter gene activity (Figures 6C and 7B). In the presence of CBP, however, a further 19-fold stimulation of reporter gene activity was observed under hypoxic conditions, whereas, under normoxic conditions, CBP only slightly (2- to 3-fold) enhanced the activity of GAL4-HIF-1 α (Figure 7B). We next wanted to examine the transactivation capacity of a GAL4 DNA binding domain fusion protein containing the C-terminal subregion 526-826 which harbors both transactivation domains of HIF-1 α and showed exclusively nuclear localization when fused to GFP (Figures 4B and 5B). This chimeric protein produced modest (about 2-fold) hypoxia-dependent activation of reporter gene activity but was potently enhanced in its inducible activation function following overexpression of CBP (Figure 7B). Thus, these data demonstrate that the C-terminus of HIF-1 α recruits CBP in a hypoxia-dependent manner. Interestingly, CBP was also able to dramatically enhance the activity of the GAL4-HIF-1 α /526-826 K719T fusion protein carrying a point mutation in the hypoxia-inducible NLS motif of HIF-1 α . In fact, the effect of CBP to support inducible transcription by either GAL4 HIF-1 α /526-826 or GAL4-HIF-1 α /526-826 K719T was very similar (Figure 7B), thus demonstrating that inactivation of the hypoxia-inducible NLS motif does not alter the ability of this domain to respond to hypoxia by coactivator recruitment and subsequent transactivation. We also examined the ability of CBP to support the function of the isolated C-terminal transactivation domain (amino acids 776-826) fused to the GAL4 DNA binding domain. Interestingly, whereas this domain mediated activation in the presence of CBP in hypoxic cells, it failed to respond to CBP following deletion of its 13 most C-terminal amino acids (Figure 7C; compare GAL4-HIF-1 α /776-826 to GAL4-HIF-1 α /776-813), strongly suggesting that these residues constitute a target for hypoxia-dependent regulation by CBP.

8. TIF2 enhances HIF-1 α activity and interacts with the receptor in a hypoxia-dependent manner in mammalian cells

5 To gain a better understanding of the mechanism of transcriptional activation by HIF-1 α , we tested the effect of transient expression of the transcriptional coactivator TIF2 on the functional activity of HIF-1 α . TIF2 belongs to the p160 family of coactivators which have been shown to be required for nuclear receptor-mediated transcriptional activation (Takeshita et al. (1997) J. Biol. Chem. 272, 27629-27634). Transient transfection experiments were
10 initially performed using COS7 cells. As shown in Fig. 8, the reporter gene activity was not significantly altered by hypoxic treatment (1% O₂) in the absence of coexpressed proteins, most probably due to the low levels of endogenous HIF-1 activity in COS7 cells (unpublished observations). Upon transient coexpression of HIF-1 α and Arnt, pT81/HRE-luc reporter gene activity was stimulated around 10-fold under normoxic conditions, possibly due
15 to a small but detectable pool of HIF-1 α localized in the nucleus under these conditions. This reporter gene activity showed a modest increase (~1.5-fold) by hypoxia (Fig. 8). However, as expected, ectopic expression of CBP further enhanced hypoxia-dependent activation by HIF-1 α in a dose-dependent manner when compared with hypoxia stimulated activity in the absence of exogenous coactivators (2- to 3-fold). Although expression of CBP slightly
20 stimulated HIF-1 α activity already at normoxia, its overall activity was almost entirely dependent on the exposure to hypoxia as has been described previously. The effect was most likely limited to 3-fold due to the large quantities of endogenous CBP already present in the cell prior to transfection. Coexpression of HIF-1 α together with TIF2 resulted in potent activation (around 12-fold) of the reporter gene over the levels observed at normoxia, thereby
25 establishing that both TIF2 and CBP support transcription by the HIF-1 α /Arnt complex (Fig. 8). In conclusion, TIF-2 which was originally identified as a coactivator supporting hormone-dependent transcriptional activation by members of the steroid hormone receptor family also functions as a conditionally regulated coactivator in hypoxia-dependent transcriptional activation by the HIF-1 α /Arnt complex.

In response to hypoxia, HIF-1 α is imported to the nucleus and shows a strictly nuclear localization. To characterize the mechanism of TIF2-dependent enhancement of transcriptional activation by HIF-1 α in living cells we next asked whether overexpression of TIF2 would affect the intracellular localization of HIF-1 α . To this end we transiently
5 transfected COS7 cells with an expression vector encoding an in-frame fusion of green fluorescent protein (GFP) with full-length HIF-1 α . As shown above, fluorescence of this GFP-HIF-1 α construct was uniformly distributed throughout the cell under normoxic conditions, and treatment of the transfected cells with the hypoxia-mimicking agent 2,2'-dipyridyl for 2 h led to a complete nuclear accumulation of the GFP-HIF-1 α protein.

10 Transient expression of the TIF2 coactivator protein in the presence of GFP-HIF-1 α had no effect either on the intensity or the subcellular distribution of fluorescence activity at normoxia. However, upon exposure of cells coexpressing GFP-HIF-1 α and TIF2 to 2,2'-dipyridyl, GFP-HIF-1 α -dependent fluorescence activity was detected in dot-like structures throughout the nucleus. It has previously been described that TIF2 is a nuclear protein mainly
15 associated with such dot-like discrete bodies within nucleus (Voegel et al. (1998) EMBO J. 17, 507-519). These observations strongly suggest that TIF-2 induced relocalization of GFP-HIF-1 α within the nucleus, indicating that GFP-HIF-1 α and TIF2 interacted with one another in vivo, and that the dot-like fluorescence pattern might represent transcriptionally active chromatin.

20 As described above, return of cells to normoxia following exposure to hypoxia or withdrawal of hypoxia-mimicking chemicals results in an export of the nuclear pool of HIF-1 α . In order to address whether the interaction between GFP-HIF-1 α and TIF2 in vivo would be dependent on the hypoxic stimulus we therefore initially exposed the transfected cells to 2,2'-
25 dipyridyl and then further incubated them at normal levels of atmospheric oxygen tension after withdrawal of 2,2'-dipyridyl by washing of the cells in medium. As expected, after 24 h of incubation under normoxic conditions, a distribution of GFP-HIF-1 α in both the nuclear and cytoplasmic compartments was observed that was indistinguishable from the distribution of fluorescence activity prior to exposure to the hypoxic signal. Transient expression of TIF2
30 did not induce any quantitative or qualitative differences in the export of GFP-HIF-1 α ,

demonstrating that the hypoxia-induced nuclear relocalization of HIF-1 α by TIF2 could be reversed following withdrawal of the hypoxic signal.

9. Regulation of the hypoxia-inducible transactivation function of HIF-1 α by SRC-1

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As outlined above, TIF2 belongs to a growing family of the p160 family of coactivators including SRC-1. Both TIF2 and SRC-1 appear to have similar activities as coactivators to enhance the transcriptional potential of many members of the ligand-activated nuclear hormone receptors (Voegel et al., *supra*). SRC-1 has been demonstrated to directly and
10 constitutively interact with CBP (Yao et al. (1996) Proc. Natl. Acad. Sci. USA 93, 10626-10631). Moreover, in analogy to CBP, SRC-1 has been shown to possess intrinsic histone acetyltransferase activity (Spencer et al. (1997) Nature 389, 194-198). Against this background we also tested the ability of SRC-1 to modulate HIF-1 α -dependent transcriptional
15 activation of the minimal HRE-driven reporter gene. In transient transfection experiments using COS7 cells, HRE-dependent reporter gene activity was stimulated from 2- to 8-fold in a dose-dependent manner by SRC-1, as compared with hypoxia-stimulated activity in the absence of exogenous coactivators (Fig. 9A). Transient expression of SRC-1 also enhanced
the transcriptional activity of HIF-1 α under normoxic conditions, albeit to a lesser extent (around 3-fold; Fig. 9 A). Thus, both SRC-1 and TIF2 have similar properties in enhancing
20 the transcriptional potential of HIF-1 α . To determine whether regulation of HIF-1 α function by members of the p160 family of coactivators was cell type-specific, we next carried out similar transfection experiments in the human 293 embryonic kidney cell line. In these experiments we observed that both SRC-1 (Fig. 9B) and TIF2 (data not shown) can significantly enhance HIF-1 α -dependent transcriptional activation in 293 cells.

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Interestingly, in analogy to HIF-1 α , the p160 family of coactivators belongs to the larger family of bHLH/PAS factors (schematically represented in Fig. 9C). SRC-1 was originally cloned as an N-terminally truncated fragment (Oñate et al. (1995) Science 270, 1354-1357), here termed SRC-1 Δ PAS (Fig. 9C), lacking the bHLH/PAS motifs. Both the bHLH and PAS
30 domains represent potent protein/protein interaction interfaces, and are critical for dimerization between HIF-1 α and Arnt (Gradin et al. (1996) Mol. Cell Biol. 16, 5221-5231;

Wang et al. (1995) Proc. Natl. Acad. Sci. USA 92, 5510-5514). To investigate the role of the bHLH/PAS motif of SRC-1 in hypoxia-dependent transactivation by HIF-1 α we examined the effect of overexpression of SRC-1 Δ PAS or full-length SRC-1 on the transcriptional activity of a fusion protein, GAL4/HIF 71-826, containing the GAL4 DBD fused in frame to a fragment of hHIF-1 α spanning amino acids 71-826. Hypoxia-dependent activation of this construct was monitored using a GAL4-responsive reporter gene. As shown in Fig. 9C, the chimeric protein mediated ~4-fold hypoxia-dependent activation of reporter gene activity. In agreement with the effect of SRC-1 on transcriptional activation mediated by the HIF-1 α /Arnt complex, coexpression of full-length SRC-1 coactivator further enhanced the activity of GAL4/HIF 71-826 by about 12-fold under hypoxic conditions, resulting in ~45-fold stimulation of the reporter gene over the levels observed at normoxia (Fig. 9C). SRC-1 showed no detectable effect on the transcriptional activity by the GAL4 DBD alone (data not shown). These results demonstrate that SRC-1 targets the HIF-1 α protein. Next, we examined the effect of the SRC-1 Δ PAS mutant on GAL4/HIF 71-826-mediated transactivation. This truncated coactivator fragment showed very similar if not more potent activity than full length SRC-1 in enhancing the hypoxia-dependent activation response (Fig. 9C). Thus, in excellent agreement with the wild-type activity of SRC-1 Δ PAS in enhancing steroid hormone receptor-dependent transcriptional regulation (Ofiate et al., *supra*), the bHLH and PAS motifs of SRC-1 were not important to support HIF-1 α function.

The present experiments indicated the functional importance of both CBP and SRC-1 to enhance the transcriptional potential by HIF-1 α in hypoxic cells. CBP has previously been demonstrated to physically interact with HIF-1 α (Arany et al. (1995) Nature 374, 81-84). To investigate whether SRC-1 was able to interact with HIF-1 α , we performed glutathione-S-transferase (GST) precipitation assays using cell extracts prepared from COS7 cells transiently expressing GST-tagged HIF-1 α in the presence and absence of 100 μ M CoCl₂, a chemical known to mimic hypoxic induction of target gene expression and to activate HIF-1 α (Wenger & Gassmann (1997) Biol. Chem. 378, 609-616). This material was subsequently incubated with in vitro translated, [³⁵S]labeled SRC-1. As shown in Fig. 9D, in the presence of CoCl₂, [³⁵S]labeled SRC-1 was specifically retained by the GST-HIF-1 α fusion protein immobilized on glutathione-Sepharose beads (lane 4), whereas we observed no significant

binding to GST-HIF-1 α extracted from non-treated cells (lane 3), or to GST alone (lane 2). Thus, SRC-1 interacted with HIF-1 α in a hypoxia-dependent manner in vitro.

10. Role of LXXLL motifs in HIF-1 α -SRC-1 interaction

SRC-1 contains four copies of the short sequence motif, LXXLL. Three of these motifs were identified in the central interaction domain of the protein, and the fourth motif was found in the eight most C-terminal amino acids of human SRC-1 (Heery et al. (1997) Nature 387, 733-736). The LXXLL motifs have been shown to be necessary to mediate the binding of SRC-1 to ligand occupied nuclear receptors. In order to investigate the role of the LXXLL motifs of SRC-1 in hypoxia-dependent transactivation by HIF-1 α we compared the abilities of wild-type SRC-1 and SRC-1 M1234, a mutant protein in which the conserved leucine couplets were substituted to alanine at residues 636/7, 693/4, 752/3 and 1438/9, respectively, in transient transfection experiments. As shown in Fig. 10, the mutant protein in which all four functional binding motifs are disabled enhanced the activity of GAL4/HIF 71-826 to the same extent as the wild-type SRC-1 under hypoxic conditions. Moreover, transient expression of either wild-type SRC-1 or SRC-1 M1234 together with GFP-HIF-1 α protein in COS7 cells had no effect on the subcellular distribution of fluorescence activity at normoxia. Under hypoxic conditions, SRC-1 induced relocalization of GFP-HIF-1 α from a uniform nuclear distribution to discrete dot-like structures within nucleus. This effect was also produced by the mutant SRC-1 M1234 protein, indicating that both proteins interact with HIF-1 α in a hypoxia-dependent manner in vivo. Taken together these results suggest that, in contrast to nuclear receptor signaling, the LXXLL motif is not required for HIF-1 α -SRC-1 interaction.

11. Definition of the functional domains of HIF-1 α which interact with the coactivators CBP and SRC-1

In order to identify HIF-1 α structures which could serve as interaction surfaces with the SRC-1 and CBP coactivator proteins, we constructed a series of GAL4 DBD fusion proteins containing HIF-1 α fragments of varying length (as schematically represented in Fig. 11A).

These constructs were transiently transfected into COS7 cells together with a GAL4 responsive reporter gene. Fusion of full length HIF-1 α to the GAL4 DBD produced significant hypoxia-dependent induction of reporter gene activity in the absence of ectopically expressed coactivator proteins (Fig. 11A). Coexpression of either CBP or SRC-1 strongly (around 8- to 10-fold) enhanced the transcriptional potential of HIF-1 α in hypoxic cells. These coactivators also increased to a lesser extent the transcriptional activity of GAL4/HIF 1-826 under normoxic conditions. Neither CBP nor SRC-1 enhanced the transcriptional activity of the GAL4 DBD alone (data not shown).

10 GAL4/HIF 71-826, which lacks the bHLH domain of HIF-1 α (The amino acid sequence of HIF-1 α /71-826 is shown as SEQ ID NO: 11), induced relative luciferase activity about 3.5-fold over background levels (Fig. 11A). In the presence of coexpressed CBP or SRC-1, luciferase activity was induced under hypoxic conditions by GAL4/HIF 71-826 to 30- and 34-fold higher levels, respectively, in comparison with the activity observed at hypoxia in the
15 absence of exogenous coactivators.

We next examined the effect of CBP and SRC-1 on the activity of GAL4 fusion proteins containing sequences lying C-terminally to the DNA binding and dimerization domains of HIF-1 α . An hypoxia-inducible activation response was produced by GAL4/HIF 526-826 alone, in excellent agreement with the presence within this HIF-1 α fragment of two distinct
20 transactivation domains, delineated N-TAD and C-TAD, respectively, in Fig. 11A, which function in a hypoxia-dependent fashion when fused to the GAL4 DBD (Jiang et al. (1997) J. Biol. Chem. 272, 19253-19260; Pugh et al. (1997) J. Biol. Chem. 11205-11214). Upon transient expression of CBP or SRC-1 the transcriptional activity of GAL4/HIF 526-826 was
25 greatly (about 7- to 9-fold) stimulated, demonstrating that these coactivators target the C-terminus of HIF-1 α . Moreover, these results further establish that the enhancing effect of both CBP and SRC-1 on HIF-1 α function is independent of the presence of the bHLH and PAS domains of HIF-1 α .

30 To further investigate whether one individual or both transcriptional activation domains of HIF-1 α were targeted by the coactivators, we next deleted residues 813-826, a region that is

contained within the C-terminal transactivation domain of HIF-1 α and has been identified as a point of interaction with CBP (Arany et al. (1996) Proc. Natl. Acad. Sci. USA 93, 12969-12973). Interestingly, GAL4/HIF 1-813 (The amino acid sequence of HIF-1 α /1-813 is shown as SEQ ID NO: 12) maintained a hypoxia-inducible response that was significantly enhanced (14- to 13-fold) by overexpression of CBP and SRC-1 (Fig. 11A), indicating that the hypoxia-dependent function of the N-terminal transactivation domain of HIF-1 α may also be regulated by the coactivators.

Against this background we were interested in examining the regulatory properties of the individual transactivation domains of HIF-1 α . As shown in Fig. 11A, GAL4/HIF 531-584 (The amino acid sequence of HIF-1 α /531-584 is shown as SEQ ID NO: 13), a chimeric protein spanning the N-terminal transactivation domain of HIF-1 α , showed hypoxia-dependent induction of reporter gene activity, albeit with a lower potency than the constructs spanning both transactivation domains. In the presence of CBP, however, an 11-fold stimulation of reporter gene activity was observed in hypoxic cells, whereas, overexpression of SRC-1 only slightly (1.5-fold) enhanced the activity of GAL4/HIF 531-584 under identical conditions.

We next examined the transactivation capacity of GAL4/HIF 526-641 which harbors both the N-terminal transactivation domain and a structure which has been proposed to function as an inhibitory sequence (Jiang et al., *supra*). In comparison with GAL4/HIF 531-584, the transcriptional activity of this chimeric protein was greatly reduced and showed complete abrogation of the effect of CBP and SRC-1 on its transactivation function (Fig. 11A), indicating either that an inhibitory region is contained between residues 584 and 641 that may serve to modulate HIF-1 α function under certain as yet unidentified conditions, or, alternatively, that the fusion of the N-terminal transactivation domain to this sequence produces a malformed domain when expressed out of the context of the full length protein.

In comparison, a fusion protein containing the carboxy-terminal transactivation domain of HIF-1 α , GAL4/HIF 776-826 (The amino acid sequence of HIF-1 α /776-826 is shown as SEQ ID NO: 14), produced a very modest (about 2-fold) hypoxia-dependent induction response.

However, its transcriptional activity was dramatically enhanced in the presence of either CBP (29-fold increase) or SRC-1 (17-fold increase). Upon deletion of the 13 most carboxy-terminal amino acids both basal activity and inducible properties of GAL4/HIF 776-813 (The amino acid sequence of HIF-1 α /776-813 is shown as SEQ ID NO: 15) were completely
5 abrogated independently of the absence or presence of coactivators (Fig. 11A).

Taken together, these results suggest that (i) CBP mediates the hypoxia-inducible transcriptional activation by HIF-1 α by targeting both of the transactivation domains of HIF-1 α , whereas SRC-1 seems to exert its action mainly through the C-terminal transactivation
10 domain of the receptor; and (ii) the 13 most C-terminal amino acids of HIF-1 α are crucial for stimulation of the activity of the C-terminal transactivation domain by CBP or SRC-1. However, in the context of the full-length protein, this C-terminal portion plays only a minor part in determining hypoxia-dependent functional activity in the presence of either CBP or SRC-1.

15 Since Arnt, the functional partner factor of HIF-1 α , has recently been shown to interact with CBP/p300 (Kobayashi et al. (1997) J. Biochem. 122, 703-710) we also examined whether SRC-1 might functionally interact with Arnt. In normoxic cells Arnt functions as a constitutively active transcription factor on E box-driven promoters. We transiently
20 expressed in COS7 cells Arnt fused to the GAL4 DBD together with either CBP or SRC-1. In these experiments we observed that constitutive activation of the reporter gene by GAL4/Arnt 128-774 was potently further enhanced by coexpression of CBP or SRC-1 (Fig. 11B). Deletion of the C-terminal transactivation domain of Arnt (schematically represented in Fig. 11B) completely abolished the effect of these coactivators on transcription by
25 GAL4/Arnt 128-603 (Fig. 11B). These observations suggest that the subunits of the HIF-1 heterodimer, HIF-1 α and Arnt, interact independently with CBP and SRC-1. Interestingly, whereas Arnt interacted constitutively with CBP and SRC-1, the functional communication of the HIF-1 α -Arnt heterodimeric complex with either CBP or SRC-1 was hypoxia-dependent. Thus, dimerization with HIF-1 α appears to impose hypoxia-dependent regulation
30 on the ability of Arnt to functionally interact with these coactivator proteins. To further examine this question, we analyzed the effect of overexpression of HIF-1 α on the activity of

GAL4/Arnt fusion proteins at normoxia. Cotransfection of COS7 cells with HIF-1 α and GAL4/Arnt 128-774 resulted in ~4-fold activation of the reporter gene when compared to cells transfected with the GAL4 fusion protein alone (Fig. 11C) probably due to the presence of small amounts of HIF-1 α in the nucleus already at normoxia. However, constitutive activation of the reporter gene was completely abolished by coexpression of HIF-1 α together with CBP or SRC-1 (Fig. 11C). This effect was indistinguishable from the one observed with GAL4/Arnt 128-603 in the presence of either of the coactivators (Fig. 11B). This deletion mutant, however, when cotransfected with HIF-1 α resulted in further ~5-fold stimulation of reporter gene activity (Fig. 11C), in agreement with its ability to functionally interact with HIF-1 α via the PAS domain. These results are consistent with the model that dimerization with HIF-1 α impairs the ability of Arnt to functionally interacting with CBP or SRC-1. However, the mechanism of this putative negative regulatory effect of HIF-1 α needs to be further investigated.

12. SRC-1 and CBP act synergistically to enhance HIF-1 α -dependent transcription

The ability of either CBP or SRC-1 to enhance the transcriptional activity of HIF-1 α prompted us to investigate the effect of coexpression of both coactivators on HIF-1 α function. We initially investigated the effects of transient expression of SRC-1 and CBP individually and in combination with one another on hypoxia-dependent activation by HIF-1 α of a minimal hypoxia response element-driven reporter gene. As observed in Figs. 9A and 2B, the effect of SRC-1 on HIF-1 α -dependent activation of the reporter gene was strictly dose-dependent in COS7 and 293 cells, respectively. For instance, at a low concentration of SRC-1 expression vector (0.75 μ g), no significant effect on HIF-1 α -dependent activation of the reporter gene was detected (Fig. 9A; 9B; 12A). In a similar fashion, an identically low concentration of CBP expression vector produced no enhancement of the transactivation function of HIF-1 α (Fig. 12A). Under all these conditions, poor hypoxia-dependent inducibility of the reporter gene was observed. However, a potent, hypoxia-dependent activation response can be reconstituted upon transient coexpression of the SRC-1 and CBP at doses which yielded no significant effect individually (Fig. 12A), indicating strong synergy between these two coactivators in enhancing HIF-1 α function.

To further substantiate this synergistic effect, we next examined the transactivation potential of a number of GAL4 DBD fusion proteins containing distinct subregions of HIF-1 α . Low concentrations of SRC-1 and CBP produced modest stimulation of the transcriptional activity by GAL4/HIF 71-826 spanning HIF-1 α lacking the N-terminal bHLH domain (Fig. 12B). In contrast, coexpression of SRC-1 and CBP generated strong enhancement of the GAL4/HIF 71-826-mediated activation response in hypoxic cells. This effect was more than additive relative to the effects detected in the presence of CBP or SRC-1 alone, and it was also observed using the GAL4/HIF 526-826 fusion protein which contains both the N-terminal and C-terminal transactivation domains of HIF-1 α (Fig. 12B). Moreover, coexpression of low concentrations of SRC-1 and CBP potently stimulated hypoxia-inducible transcriptional activation by the individual transactivation domains of HIF-1 α contained within GAL4/HIF 531-584 and GAL4/HIF 776-826, respectively. These results are in excellent agreement with the data demonstrating that the two transactivation domains of HIF-1 α independently can functionally interact with either SRC-1 or CBP (Fig. 11A). These data also are consistent with the notion that CBP and SRC-1 constitutively interact with one another, and, in analogy to the mechanism of transcriptional activation by members of the steroid hormone receptor family, that recruitment of both coactivators may be necessary to trigger the full activity of HIF-1 α in hypoxic cells. It remains to be established whether the recruitment of both of these classes of coactivators to HIF-1 α occurs simultaneously or in a temporally regulated fashion. Furthermore it is presently unclear whether CBP, SRC-1 or both coactivators provide the physical contact points with the transactivation domains of HIF-1 α upon hypoxic activation. Interestingly, the functional interaction between HIF-1 α and the two coactivator proteins was synergistic when both transactivation domains of HIF-1 α were contained within the analyzed GAL4-HIF-1 α fusion proteins. In contrast, GAL4 fusion proteins spanning the isolated transactivation domains showed an additive mode of regulation in the presence of both SRC-1 and CBP, strongly suggesting that synergistic regulation by these two coactivators may require the presence and integrity of both transactivation domains of HIF-1 α .

CBP and p300 are closely related proteins that exhibit strong sequence similarity and similar functions with respect to their roles as coactivators (Arany et al. (1994) Cell 77, 799-800). In

analogy to CBP, p300 enhanced in the presence of SRC-1 hypoxia-dependent transcriptional activation of reporter genes by GAL4 fusion proteins containing either one of the two individual transactivation domains of HIF-1 α (Fig. 12C). As observed in experiments using CBP (Fig. 12B), the CBP/p300 and SRC-1 classes of coactivators produced together rather modest (3- to 4-fold) hypoxic activation of the N-terminal transactivation domain located between residues 531 and 584 of HIF-1 α (Fig. 12C), whereas, in the presence of SRC-1, CBP or p300 more potently (around 11-fold) stimulated hypoxic activation by the C-terminal transactivation domain of HIF-1 α (Fig. 12C).

Although the mechanism of action of the CBP/p300 class of coactivators remains largely unclear, recent observations have suggested that these proteins contribute to transcriptional regulation not only by acting as simple adaptors between DNA binding factors and transcription initiation factors but also by harboring intrinsic histone acetyltransferase activity, linking their effect to regulation of chromatin structure and/or function (Korzus et al. (1998) Science 279, 703-707). To investigate the potential role of the acetyltransferase activity of p300 in supporting HIF-1 α -dependent transcription, we transfected COS7 cells with a p300 deletion mutant, p300 Δ HAT, lacking the histone acetyltransferase (HAT) domain which is centrally located in the protein (schematically represented in the right panel of Fig. 12C). In the absence of SRC-1, transient expression of p300 Δ HAT resulted in only very moderate (2-fold) stimulation of the hypoxia-dependent activation response produced by GAL4/HIF 531-584 containing the N-terminal transactivation domain of HIF-1 α , whereas it did not produce any significant effect on the hypoxia-induced transcriptional activity of GAL4/HIF 776-826 spanning the minimal C-terminal transactivation domain (Fig. 12C). In the presence of SRC-1, p300 Δ HAT enhanced reporter gene activation, most notably when coexpressed in hypoxic cells together with GAL4/HIF 776-826 containing the C-terminal transactivation domain of HIF-1 α . However, p300 Δ HAT was about 2-fold less potent than wild-type p300 in producing this response (Fig. 12C), indicating that the acetyltransferase catalytic activity of p300 may contribute to trigger the full activity of the HIF-1 α -mediated transcriptional activation response. However, given the observed synergy between CBP/p300 and SRC-1 in enhancing activation potency by HIF-1 α , and the fact that SRC-1 also harbors intrinsic histone acetyltransferase activity (Spencer et al. (1997) Nature 389, 194-198), the

effect of the deletion of the acetyltransferase domain of p300 may be masked by corresponding activities of CBP-associated proteins, possibly that of SRC-1 itself.

13. Ref-1 potentiates HIF-1 α function in the presence of CBP and SRC-1

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The nuclear redox regulator Ref-1 is known to stabilize the DNA binding activity of AP-1 by reduction of a conserved cysteine residue of Fos and Jun. Ref-1 is a bifunctional enzyme: it harbors both redox and endonuclease DNA repair activities (Xanthoudakis et al. (1992) EMBO J. 11, 3323-3335) and has been implicated in up-regulation of HIF-1 α -dependent induction of gene expression under hypoxic conditions (Huang et al. (1996) J. Biol. Chem. 271, 32253-32259). Against this background we wanted to further investigate the effect of Ref-1 on HIF-1 α -dependent transcriptional activation, and to examine its effect on the functional interaction of HIF-1 α with the coactivators CBP and SRC-1. To investigate whether HIF-1 α is a target of regulation by Ref-1 we initially used the different GAL4 DBD fusion proteins containing distinct subfragments of HIF-1 α . As shown in Fig. 13A, transient overexpression of Ref-1 markedly potentiated hypoxic induction of reporter gene expression by the fusion proteins GAL4/HIF 71-826 and GAL4/HIF 526-826 spanning both transactivation domain of HIF-1 α . The transcriptional activation function of GAL4/HIF 531-584 containing the N-terminal transactivation domain of HIF-1 α was only very moderately but significantly stimulated by Ref-1 in hypoxic cells, whereas Ref-1 produced more potent regulation of hypoxia-inducible transactivation by GAL4/HIF 776-826 containing the C-terminal transactivation domain (Fig. 13A).

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Given the striking potentiation of HIF-1 α function by the coactivators CBP and SRC-1 we next examined the effect of Ref-1 on transcriptional activation by the GAL4-HIF-1 α fusion proteins in combination with CBP, SRC-1, or both proteins. Following transient expression of Ref-1 together with either CBP or SRC-1 hypoxia-inducible transcriptional activation by the tested GAL4-HIF-1 α fusion proteins was not significantly altered in comparison to the results obtained in the presence of the fusion proteins and Ref-1 alone (Fig. 13B).

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Remarkably, however, the hypoxia-inducible transcriptional potencies of all the fusion proteins containing either both or the individual HIF-1 α transactivation domains were

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dramatically (10- to 53-fold) enhanced by coexpression of Ref-1 in the presence of CBP and SRC-1 (Fig. 13B). Under these conditions, the fusion proteins spanning the N- or C-terminal transactivation domains produced 12- or 53-fold hypoxia-dependent activation responses, respectively, whereas the fusion protein containing the C-terminal transactivation domain lacking the CBP interaction interface, GAL4/HIF 776-813, showed no regulation by Ref-1 and the coactivators. Thus, these data indicate that these two coactivators may have been limiting under these conditions for the Ref-1-mediated effect on HIF-1 α -dependent transcription. Moreover, these results clearly demonstrate that both the N-terminal and C-terminal transactivation domains HIF-1 α are targets of regulation by Ref-1, implying that Ref-1 together with the CBP and SRC-1 classes of transcriptional coactivators plays a key role in regulation of HIF-1 α function. Moreover, this is the first example of a transactivation domain representing a target of regulation by Ref-1, and, conversely, these data represent the first example of a noncovalent protein modifier of the HIF-1 α transactivation domains identified in mammalian cells.

The precise mechanism by which Ref-1 activates HIF-1 α is not known. Interestingly, a GFP-tagged Ref-1 fusion protein was exclusively localized in the nucleus both under normoxic and hypoxic conditions indicating that nuclear translocation of HIF-1 α is required for functional interaction with Ref-1. This redox regulator protein has been shown to form complexes with Jun in vitro (Xanthoudakis et al. (1994) Proc. Natl. Acad. Sci. USA 91, 23-27). To further investigate whether Ref-1 could interact with HIF-1 α , we tried to trap the interaction by using a crosslinking reagent such as diamide, which oxidizes cysteine sulfhydryls to disulfides. A series of experiments was performed with GST-tagged purified Ref-1. [³⁵S]methionine-labeled in vitro translated GAL4/HIF 1-826 was incubated with GST/Ref-1 in the presence or absence of diamide. GAL4/HIF 1-826 was found to weakly bind GST/Ref-1 and the crosslinking agent stabilized this interaction between Ref-1 and GAL4/HIF. No binding of [³⁵S]methionine-labeled GAL4/HIF was observed when it was incubated either with the anti-GST affinity gel alone in the absence of GST/Ref-1 or when it was incubated with purified GST, indicating that there was no significant background binding.

Next, we tried a series of pull-down experiments with [³⁵S]methionine-labeled in vitro translated GAL4/HIF 776-826 and GAL4/HIF 531-584. GAL4/HIF 531-584 bound GST/Ref-1, and no effect of diamide was observed, consistent with the absence of cysteine residues in the N-terminal transactivation domain of HIF-1 α . An interaction between GAL4/HIF 726-826 and GST/Ref-1 was also detected, and this interaction was enhanced in the presence of diamide. In excellent agreement with this observation the C-TAD contains two cysteine residues. Low levels of background binding of the labeled proteins to GST was observed in the presence or absence of diamide. Immunoblot analysis verified that the input concentrations of GST/Ref-1 or GST alone were similar.

The experiments above established that Ref-1 potentiates the effect of both the CBP and SRC-1 coactivators on hypoxia-inducible promoter activation by HIF-1 α . Moreover, Ref-1 functionally and physically interacted with the distinct N- and C-terminal transactivation domains of HIF-1 α . However, only the C-terminal and not the N-terminal transactivation domain of HIF-1 α contains cysteine residues through which Ref-1 regulation occurs (Xanthoudakis et al. (1992) EMBO J. 11, 3323-3335). These data indicate that an auxiliary factor, possibly a coactivator, may mediate interaction with at least the N-terminal transactivation domain of HIF-1 α .

MATERIALS AND METHODS

(a) Reagents

pCMX-SAH/Y145F expression vector encoding a modified and highly chromophoric form of GFP under the control of cytomegalovirus (CMV) immediate early promoter was a generous gift from Dr. Kazuhiko Umesono (Kyoto University, Japan). This humanized GFP (SAH/Y145F) contains a S65A mutation which confers a wavelength shift and temperature resistance to the protein as well as a Y145P substitution increasing the intracellular stability of GFP. pRc/RSV-mCBP-HA construct expressing full-length mouse CBP was a gift from Dr. Richard H. Goodman (Vollum Institute, Oregon). The vector expressing a GFP-fusion of

full-length HIF-1 α was generated by cutting the HIF-1 α coding region from pGEX-4T3-HIF-1 α as a *Bam*HI/*Not*I fragment (*Not*I site filled-in with Klenow polymerase) and ligating this in-frame into *Bam*HI/*Nhe*I opened pCMX-SAH/Y145F where the *Nhe*I site had been blunt-ended with Klenow. Deletion mutant HIF-1 α /1-652 was assembled by inserting a
5 *Bam*HI/*Spe*I fragment of HIF-1 α into *Bam*HI/*Nhe*I-digested pCMX-SAH/Y145F. GFP fusions encoding HIF-1 α subfragments (corresponding to amino acids 331-641, 526-641, and 526-826) were generated by amplifying the equivalent DNA sequences by PCR using Pfu DNA polymerase (Stratagene) together with primer pairs carrying *Bam*HI or *Nhe*I ends. The resulting products were inserted into *Bam*HI/*Nhe*I opened pCMX-SAH/Y145F. GFP fusions
10 carrying amino terminal sequences HIF-1 α (1-74, 1-245, 1-330) were cut out from corresponding bacterial GST fusion expression vectors and inserted in frame to *Bam*HI/*Nhe*I site (*Nhe*I blunt-ended) of pCMX-SAH/Y145F. GFP-HIF-1 α fusion proteins ending at amino acid 813 were generated by cleaving the C-terminal 13 amino acids by *Pst*I digestion. Corresponding GAL4 DNA-binding domain fusion proteins were assembled by cleaving the
15 HIF-1 α inserts as *Bam*HI/*Nhe*I fragments and religating them into pCMX expression vector.

Site-directed mutagenesis of the C-terminal NLS was performed by overlap PCR (Ausubel *et al.*, 1994) where the desired mutation (codon 719 AAG ->ACA) was introduced into a PCR product and then inserted as an *Eco*RI-*Pst*I subfragment into pGFP-HIF-1 α /526-826. A GFP
20 fusion of full-length HIF-1 α carrying K719T mutation was thereafter assembled by inserting the aminoterminal *Bam*HI-*Spe*I fragment of HIF-1 α into pGFP-HIF-1 α /526-826 K719T. PCR-based mutagenesis with specific oligonucleotides was employed to generate mutations to the N-terminal NLS motif. Fidelity of PCR reactions and identity of constructs was confirmed by sequencing the inserts.

25

(b) Cell Culture and Transfections

COS7 cells (from ATCC) were routinely maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum plus penicillin (50 IU/ml) and
30 streptomycin (50 μ g/ml). For analysis of nuclear translocation of HIF-1 α in living cells, we transiently expressed GFP-tagged HIF-1 α and its various mutant plasmids in COS7 cells. The

cells were cultured on the silane-coated cover slips in 6-cm diameter plastic dishes, and the medium was changed to OPTI-MEM medium lacking phenol red (Life Technologies, Inc.) before transfection. A plasmid cocktail containing 6 μ g of the expression plasmids for GFP-tagged HIF-1 α and its various mutants was mixed with 12 μ l of TransIT-LT1 reagent
5 (Panvera Corp., Madison, WI) and added to the culture. After 6 h of incubation, the medium was replaced with DMEM with 10% FCS. Cells were induced 24 h later with 1% O₂, 100 μ M 2,2'-dipyridyl or 100 μ M CoCl₂ or treated with vehicle only.

Transcriptional activity of GFP and GAL4 fusion constructs was analyzed in a cotransfection
10 assay where effector plasmids (0.2 μ g/ 30-mm dish) together with a reporter gene (1.5 μ g/ 30-mm dish) were introduced into COS7 or HeLa cells. The reporter plasmid encoded firefly luciferase gene under the control of thymidine kinase minimal promoter and either five or three copies of GAL4 or HIF-responsive elements, respectively.

15 *(c) Immunocytochemical analysis*

COS7 cells grown on fibronectin-coated cover-slips were transiently transfected with the expression plasmid for full-length HIF-1 α (pCMV-HIF-1 α) or HIF-1 α fused to the GAL4 DNA binding domain (pCMX-GAL4-HIF-1 α). After various treatments, the cells were fixed
20 with 4% paraformaldehyde in PBS at room temperature for 30 min. Immunostaining of the cells was carried out using anti-HIF-1 α antiserum (Kallio et al. (1997) Proc. Natl. Acad. Sci. USA 94, 5667-5672) or anti-GAL4-DBD antiserum (Upstate Biotechnology) and the appropriate biotinylated-second antibodies and streptoavidin-conjugated fluorescein isothiocyanate or Texas Red (Amersham). The cover-slips were mounted on glass slides and
25 subjected to microscopic analysis.

(d) Visualization of intracellular trafficking of GFP-tagged proteins in living cells

Transiently expressed GFP was expressed at detectable levels between 24 and 72 h after
30 transfection. Routinely, cells were used for further experiments 48 h after transfection. After various treatments, cells were examined using a Zeiss Axiovert 135 microscope enclosed by

an incubator and equipped with a heating-stage, an FITC-filter set, and epifluorescence with illumination from a Gixenon burner (Carl Zeiss Jena GmbH, Jena, Germany). Photographs were taken using Kodak Ektachrome 400, and semi-quantitative assessment of the subcellular localization of the GFP-tagged proteins was performed according to the methods described by Ylikomi et al. ((1992) EMBO J. 11, 3681-3694). In brief, subcellular localization of GFP-tagged proteins was performed by a blinded observer, counting approximately 200 cells in which GFP fluorescence was detected. The GFP fluorescence-positive cells were classified into four different categories: $N < C$ for cytoplasmic dominant fluorescence; $N = C$, cells having equal distribution of fluorescence in the cytoplasmic and nuclear compartments; $N > C$ for nuclear-dominant fluorescence; and N for exclusive nuclear fluorescence.

(e) Immunoblotting and Detection

For the detection of the HIF-1 α fusion protein expression, whole cell extracts were prepared essentially as described (Kallio et al. (1997) Proc. Natl. Acad. Sci. USA 94, 5667-5672). Briefly, cells were harvested in TEN buffer (40 mM Tris-HCl, pH 7.9; 10 mM EDTA; 150 mM NaCl). The cell pellet was frozen in liquid nitrogen and thawed by resuspending in 80 μ l of cell extraction buffer (10 mM Hepes, pH 7.9; 400 mM NaCl; 0.1 mM EDTA; 5% (v/v) glycerol; 1 mM dithiotreitol; 1 mM phenylmethylsulfonyl fluoride), followed by centrifugation for 30 min at maximal velocity. Fifty μ g of the total cell proteins were blotted after SDS polyacrylamide gel electrophoresis onto nitrocellulose filter and blocked overnight with 5 % nonfat milk in PBS. Anti-HIF-1 α antiserum was used as a primary antibody as 1:500 dilution in PBS containing 1% nonfat milk for 2 hours. After washes, 1:750 dilution of anti-rabbit IgG- horseradish peroxidase conjugate (Amersham) in PBS / 1 % nonfat milk was used as a secondary antibody. After extensive washing with PBS the complexes were visualized using enhanced chemiluminescence (Amersham) according to the manufacturer's instructions.

TABLE I

Subcellular distribution of GFP-HIF-1 α chimeras. Cells were transfected as duplicates with indicated fusion constructs and 24 h after transfection induced with 100 μ M 2,2'-dipyridyl or with vehicle only for 6 h before microscopic observation and cell counting. Cells were classified into four categories: N, cells containing exclusively nuclear fluorescence; N>C, cells in which the nuclear fluorescence exceeds the cytoplasmic fluorescence; N = C, cells having equal distribution of fluorescence; and N<C, cells having cytoplasmic fluorescence exceeding that in the nucleus. A total of 200 to 300 cells were analyzed for the distribution of fluorescence and the percentage of cells belonging to each category is indicated in the table.

	Normoxia				Hypoxia (Dipyridyl)			
	N	N>C	N=C	N<C	N	N>C	N=C	N<C
GFP	0	0	100	0	0	0	100	0
GFP-HIF-1 α	8	27	65	0	90	7	3	0
GFP-HIF-1 α /1-74	69	20	11	0	56	35	9	0
GFP-HIF-1 α /1-245	36	38	26	0	31	43	26	0
GFP-HIF-1 α /1-330	0	9	91	0	0	5	95	0
GFP-HIF-1 α /Δ178-390	57	23	20	0	42	29	29	0
GFP-HIF-1 α /331-641	0	0	100	0	0	0	100	0
GFP-HIF-1 α /526-641	0	0	100	0	0	0	100	0
GFP-HIF-1 α /1-652	0	0	0	100	0	0	0	100
GFP-HIF-1 α /526-826	100	0	0	0	100	0	0	0
GFP-HIF-1 α /526-813	90	10	0	0	83	11	3	0

TABLE II

Subcellular distribution of GFP-HIF-1 α chimeras carrying point mutations in the NLS motifs. Cells were transfected as duplicates with indicated fusion constructs and 24 h after transfection induced with 100 μ M 2,2'-dipyridyl or with vehicle only for 6 h before microscopic observation and cell counting. Cells were classified into four categories as outlined in Table I. A total of 200 to 300 cells were analyzed for the distribution of fluorescence and the percentage of cells belonging to each category is indicated in the table.

	Normoxia				Hypoxia (Dipyridyl)			
	N	N>C	N=C	N<C	N	N>C	N=C	N<C
GFP-HIF-1 α K719T	0	0	4	96	0	0	19	81
GFP-HIF-1 α /R18A	37	15	48	0	82	6	12	0
GFP-HIF-1 α R17G/R18G	50	24	26	0	84	8	18	0
GFP-HIF-1 α / Δ 178-390K719T	0	0	18	82	0	0	15	85
GFP-HIF-1 α / 526-826K719T	26	24	50	0	15	14	71	0

CLAIMS

1. A method for identifying compounds capable of modulating the function of a functional domain of human HIF-1 α , said method comprising
 - 5 (i) contacting a candidate compound with a variant of human HIF-1 α , said variant essentially lacking at least one functional domain of human HIF-1 α , or having a mutation making at least one functional domain of human HIF-1 α essentially inactive, said functional domain or domains being selected from the group consisting of
 - 10 (a) the PAS-B domain located in human HIF-1 α between amino acids 178 and 390,
 - (b) the C-terminal nuclear localization sequence (NLS) located in human HIF-1 α essentially at amino acids 718 to 721, and
 - (c) the transactivator domain (N-TAD) located in human HIF-1 α essentially between amino acids 531 to 584, and
 - 15 (d) the transactivator domain (C-TAD) located in human HIF-1 α essentially between amino acids 813 and 826, and
 - (ii) determining the effect of the candidate compound on the said variant.
2. The method according to claim 1, comprising
 - 20 (i) contacting a candidate compound with a cell expressing a said variant of human HIF-1 α , said variant being conjugated to a molecular probe, and
 - (ii) detecting the localization of the said molecular probe within the cell.
3. The method according to claim 2 wherein the said molecular probe is a fluorescent probe, and wherein a change in the relative fluorescence of the nucleus to the cytoplasm is indicative for a compound capable of modulating the function of the said functional domain.
 - 25
4. The method according to claim 3 wherein the said molecular probe is *Aequorea victoria* Green Fluorescent Protein, or a variant thereof.
 - 30

5. The method according to claim 1 wherein the said variant of human HIF-1 α is regulating the activity of a reporter gene; a change in the activity of the reporter gene being indicative of a compound capable of modulating the function of the said functional domain.
- 5 6. The method according to claim 5 wherein the said variant of human HIF-1 α is fused to the DNA binding domain of GAL4, and wherein said cells contain a GAL4 response element operatively linked to a reporter gene.
- 10 7. The method according to claim 6 wherein the said variant of human HIF-1 α is fused to the *Aequorea victoria* Green Fluorescent Protein, and wherein said cells contain an erythropoietin hypoxia response element operatively linked to a reporter gene.
- 15 8. The method according to claim 6 or 7 wherein the said reporter gene is a firefly luciferase gene.
9. The method according to any one of claims 1 to 8, carried out in the presence of a transcriptional coactivator.
- 20 10. The method according to claim 9, wherein the said transcriptional coactivator is chosen from the group consisting of SRC-1, TIF2 and CBP.
11. The method according to claim 9 or 10, carried out in the presence of the redox regulatory protein Ref-1.
- 25 12. The method according to any one of claims 1 to 11 wherein the said functional domain of human HIF-1 α is the PAS-B domain.
13. The method according to claim 12 wherein the said variant of human HIF-1 α is
30 HIF-1 α /1-74 (SEQ ID NO: 2),
HIF-1 α /1-245 (SEQ ID NO: 3),

HIF-1 α /Δ178-390 (SEQ ID NO: 6),
HIF-1 α /331-641 (SEQ ID NO: 7),
HIF-1 α /526-641 (SEQ ID NO: 8),
HIF-1 α /526-813 (SEQ ID NO: 9), or
5 HIF-1 α /526-826 (SEQ ID NO: 10).

14. The method according to claim 1 to 11 wherein the said functional domain of human HIF-1 α is the C-terminal nuclear localization sequence (NLS) domain.

10 15. The method according to claim 14 wherein the said variant of human HIF-1 α is
HIF-1 α /1-74 (SEQ ID NO: 2),
HIF-1 α /1-245 (SEQ ID NO: 3),
HIF-1 α /1-330 (SEQ ID NO: 4),
HIF-1 α /1-652 (SEQ ID NO: 5),
15 HIF-1 α /331-641 (SEQ ID NO: 7), or
HIF-1 α /526-641 (SEQ ID NO: 8).

16. The method according to claim 1 to 11 wherein the said functional domain of human HIF-1 α is the transactivator domain (N-TAD) located in human HIF-1 α essentially
20 between amino acids 531 to 584.

17. The method according to claim 16 wherein the said variant of human HIF-1 α is
HIF-1 α /1-74 (SEQ ID NO: 2),
HIF-1 α /1-245 (SEQ ID NO: 3),
25 HIF-1 α /1-330 (SEQ ID NO: 4),
HIF-1 α /776-826 (SEQ ID NO: 14), or
HIF-1 α /776-813 (SEQ ID NO: 15).

18. The method according to claim 1 to 11 wherein the said functional domain of human HIF-1 α is the transactivator domain (C-TAD) located in human HIF-1 α essentially between amino acids 813 and 826.
- 5 19. The method according to claim 18 wherein the said variant of human HIF-1 α is
HIF-1 α /1-74 (SEQ ID NO: 2),
HIF-1 α /1-245 (SEQ ID NO: 3),
HIF-1 α /1-330 (SEQ ID NO: 4),
HIF-1 α /1-652 (SEQ ID NO: 5),
10 HIF-1 α /331-641 (SEQ ID NO: 7),
HIF-1 α /526-641 (SEQ ID NO: 8),
HIF-1 α /526-813 (SEQ ID NO: 9),
HIF-1 α /1-813 (SEQ ID NO: 12),
HIF-1 α /531-584 (SEQ ID NO: 13),
15 HIF-1 α /776-813 (SEQ ID NO: 15).
20. An isolated variant of human HIF-1 α , said variant being selected from the group consisting of
HIF-1 α /1-74 (SEQ ID NO: 2),
20 HIF-1 α /1-245 (SEQ ID NO: 3),
HIF-1 α /1-330 (SEQ ID NO: 4),
HIF-1 α /1-652 (SEQ ID NO: 5),
HIF-1 α /Δ178-390 (SEQ ID NO: 6),
HIF-1 α /331-641 (SEQ ID NO: 7),
25 HIF-1 α /526-641 (SEQ ID NO: 8),
HIF-1 α /526-813 (SEQ ID NO: 9),
HIF-1 α /526-826 (SEQ ID NO: 10),
HIF-1 α /71-826 (SEQ ID NO: 11),
HIF-1 α /1-813 (SEQ ID NO: 12),
30 HIF-1 α /531-584 (SEQ ID NO: 13),

HIF-1 α /776-826 (SEQ ID NO: 14), and
HIF-1 α /776-813 (SEQ ID NO: 15).

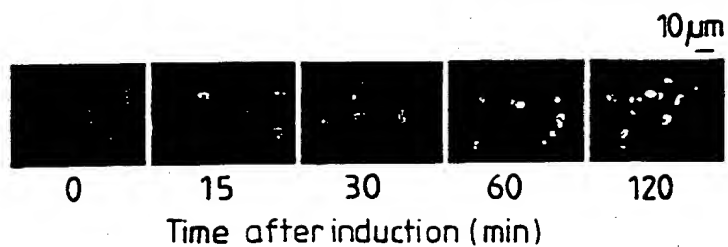
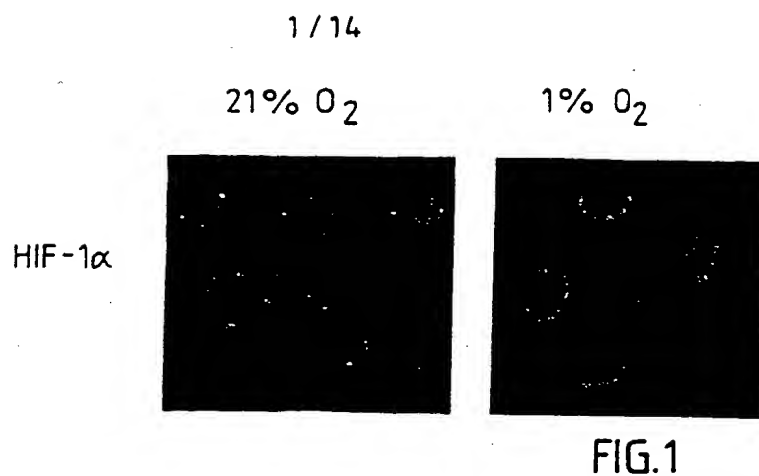


FIG.2D

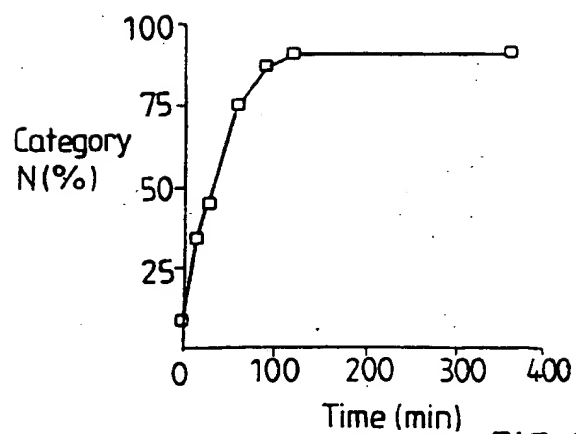
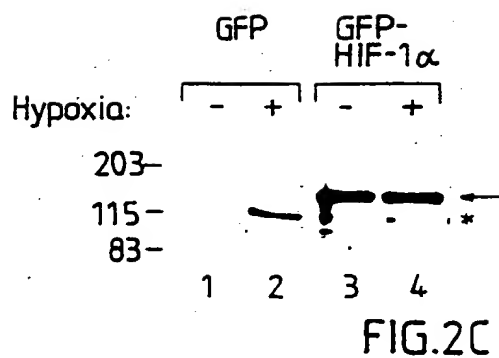
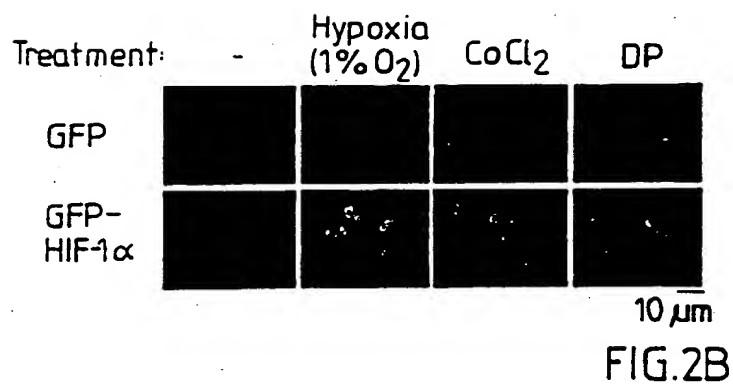
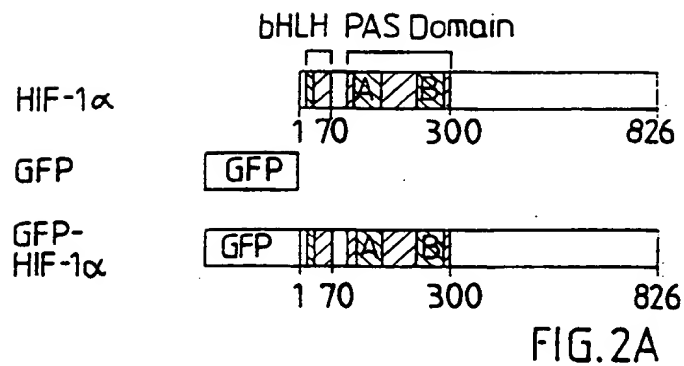


FIG. 2E

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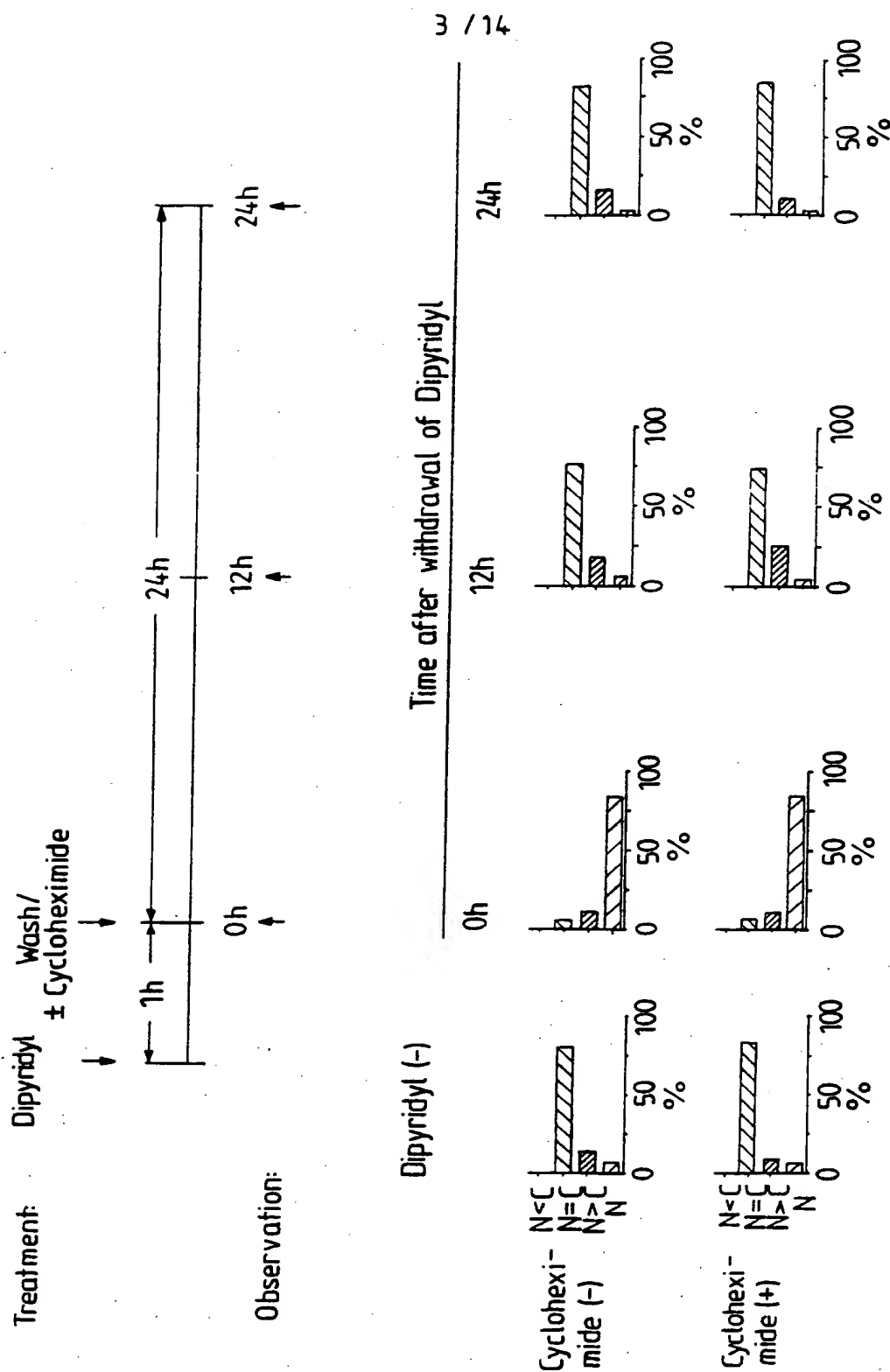


FIG. 3

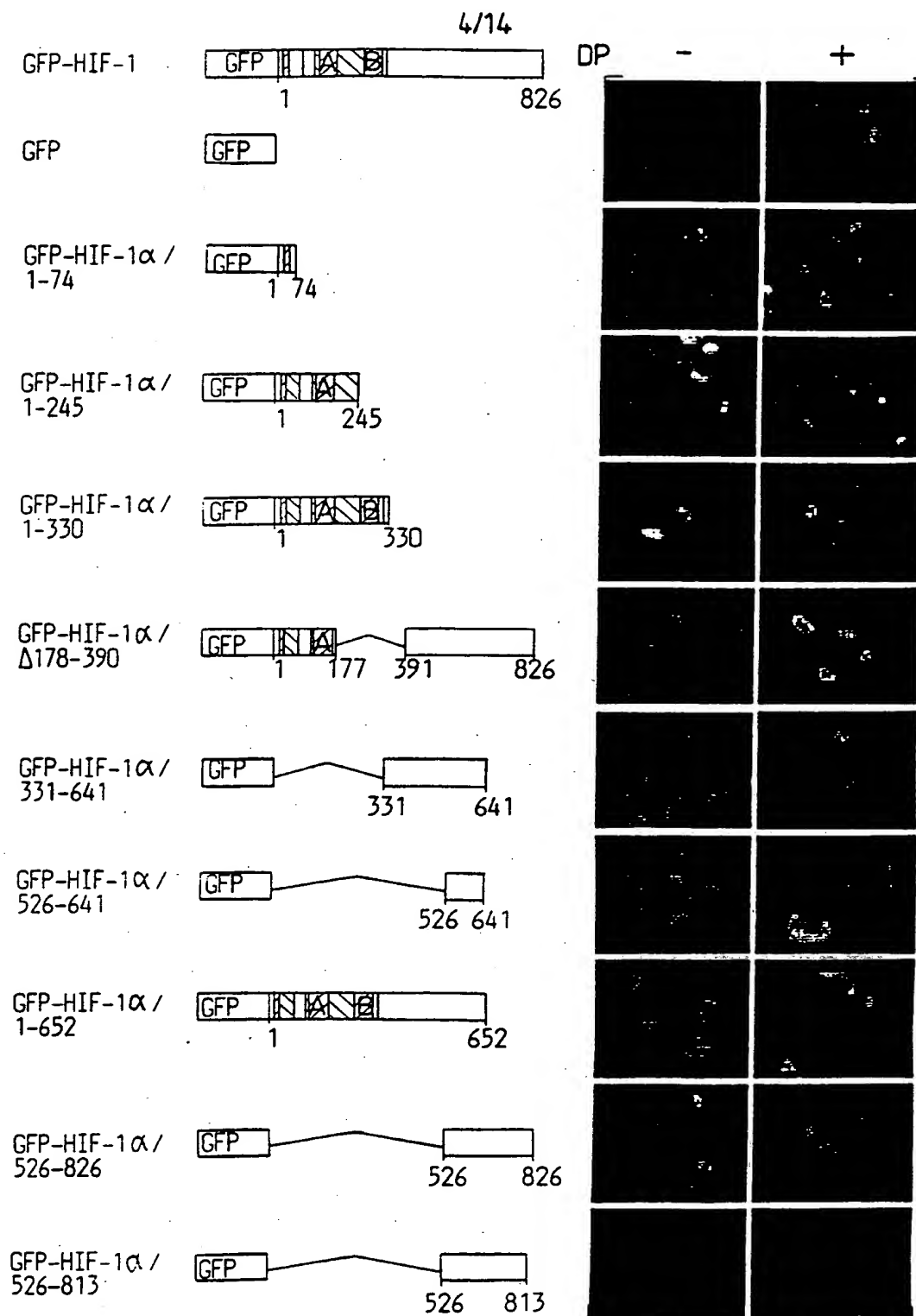
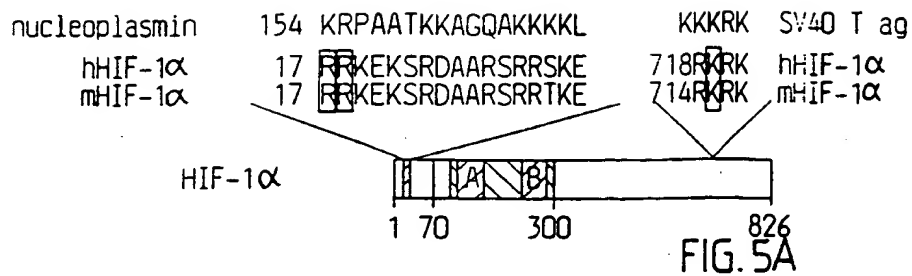


FIG. 4A

FIG. 4B

10μm

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DP:

- +

GFP-HIF-1 α

GFP-HIF-1 α
K719T

GFP-HIF-1 α
R18A

GFP-HIF-1 α
R17G/R18G

GFP-HIF-1 α /
 Δ 178-390

GFP-HIF-1 α /
 Δ 178-390 K719T

GFP-HIF-1 α /
526-826

GFP-HIF-1 α /
526-826 K719T

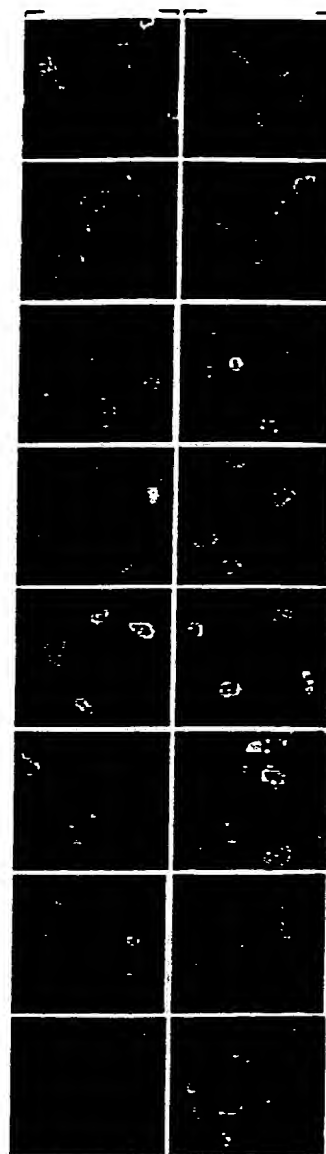
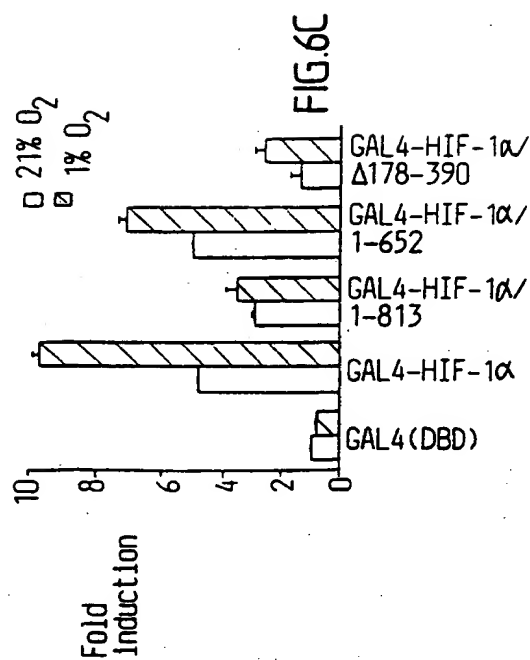
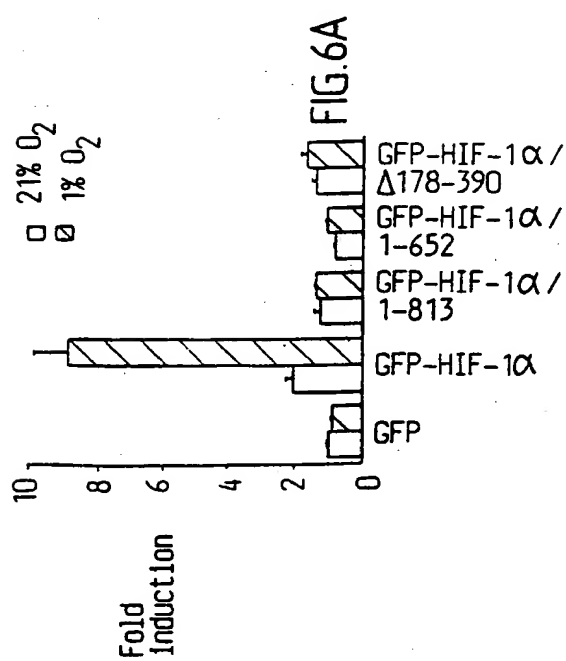
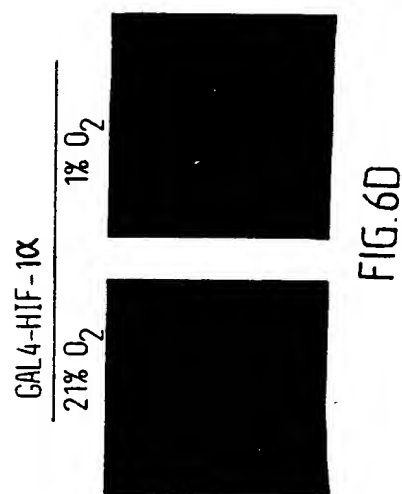
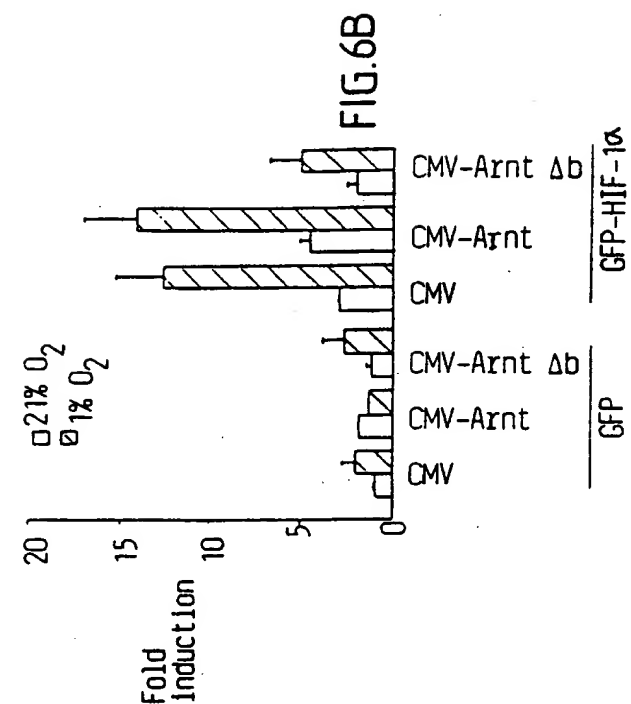
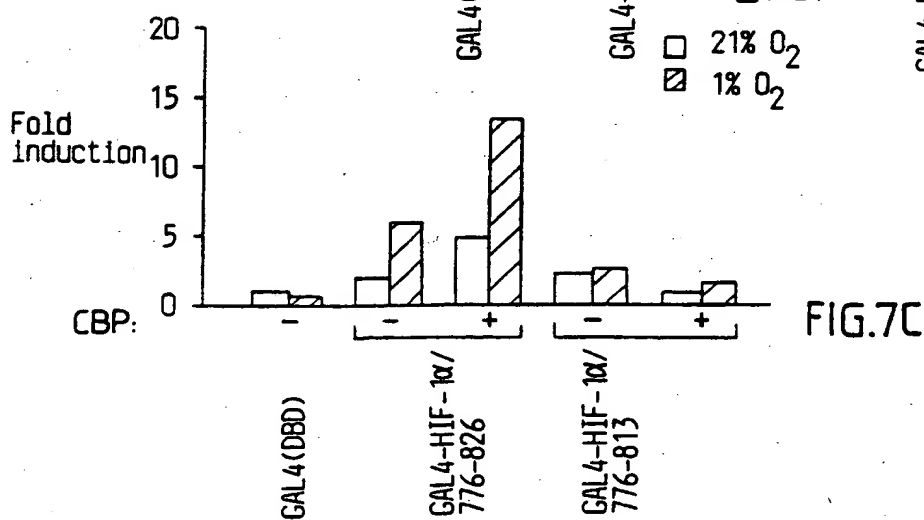
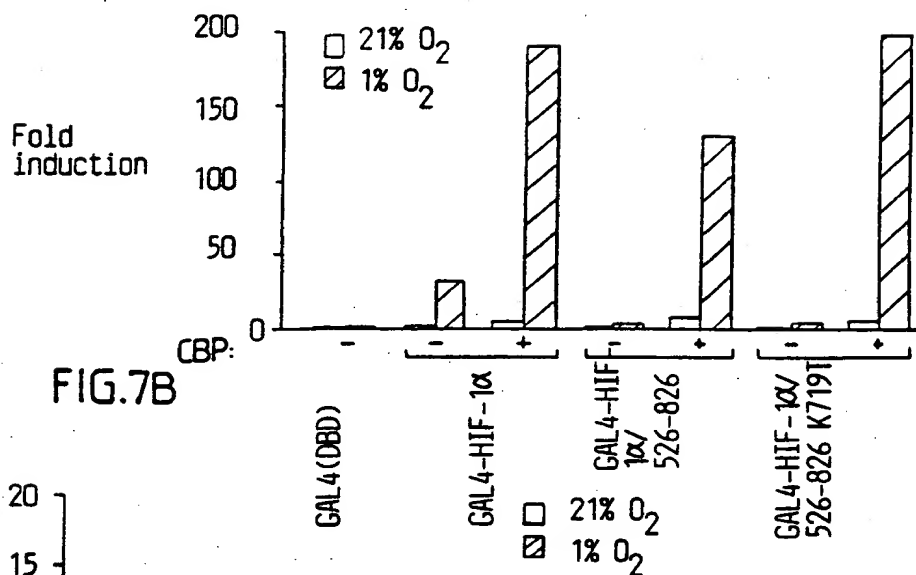
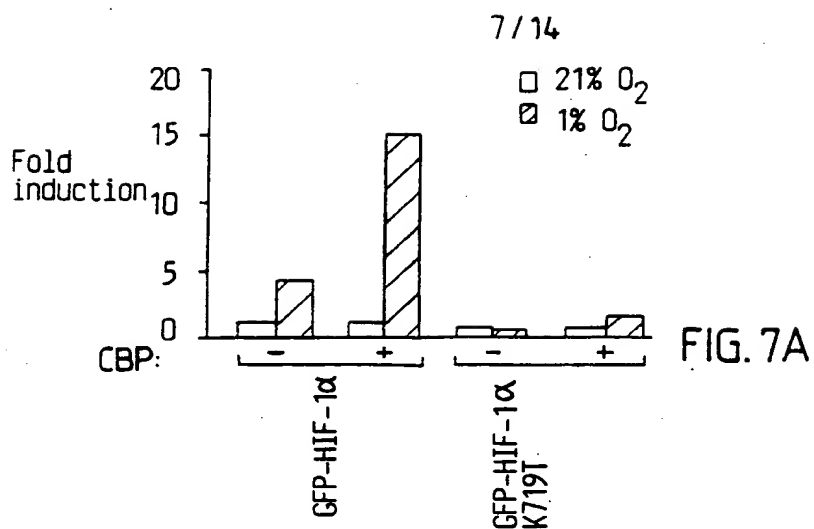


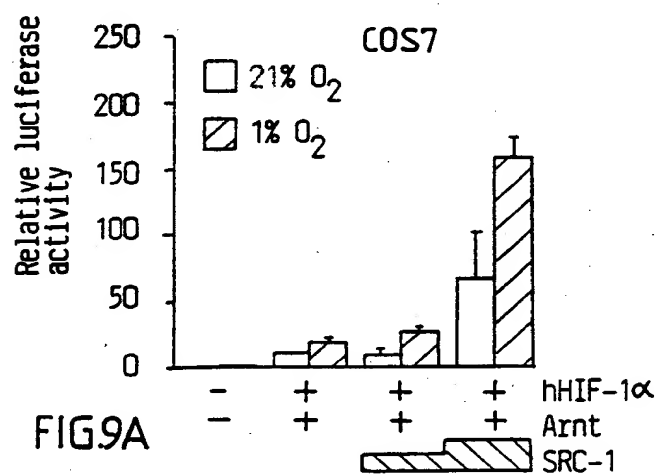
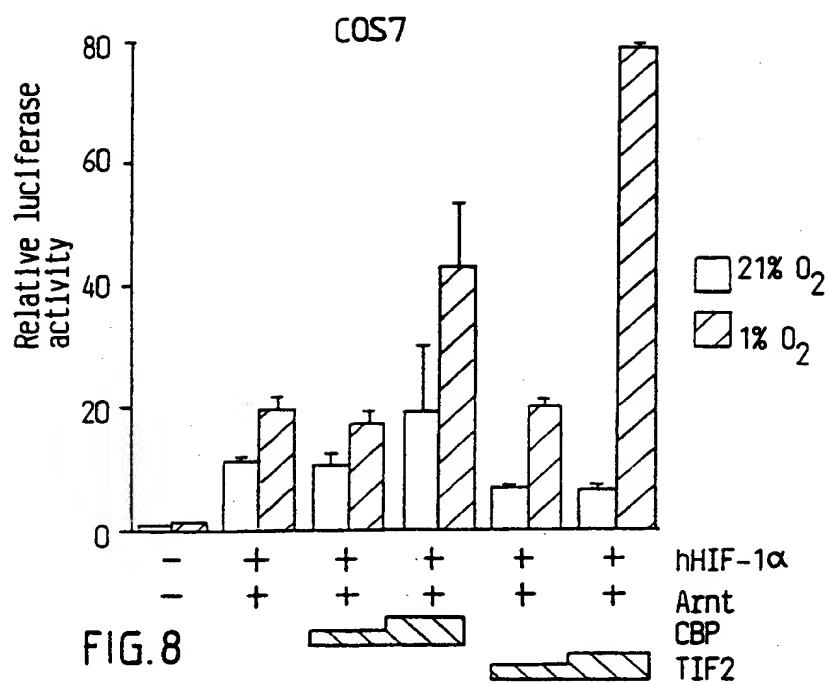
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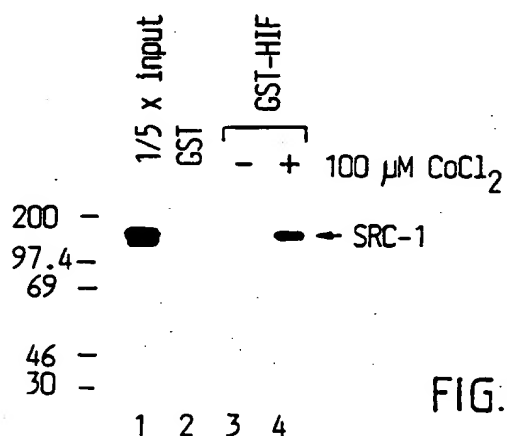
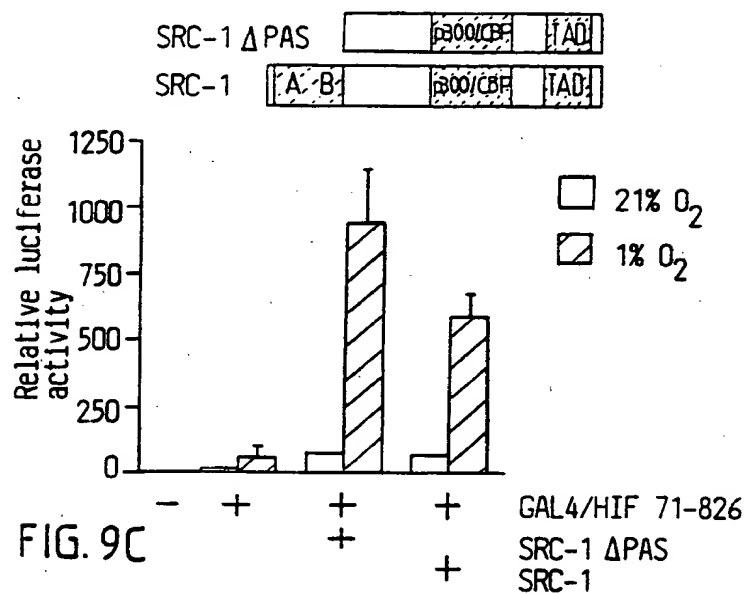
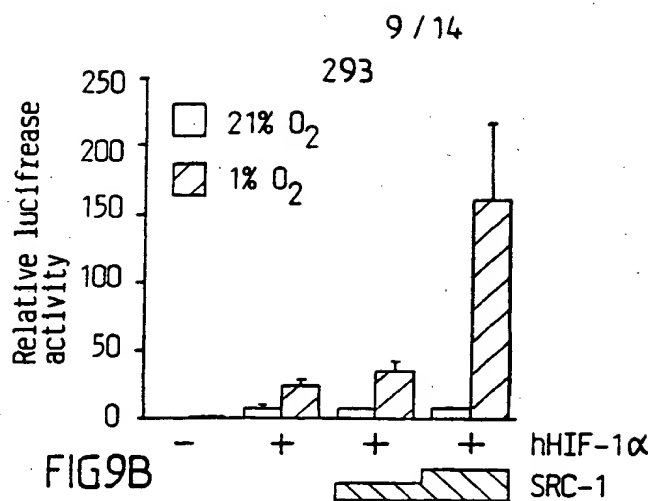
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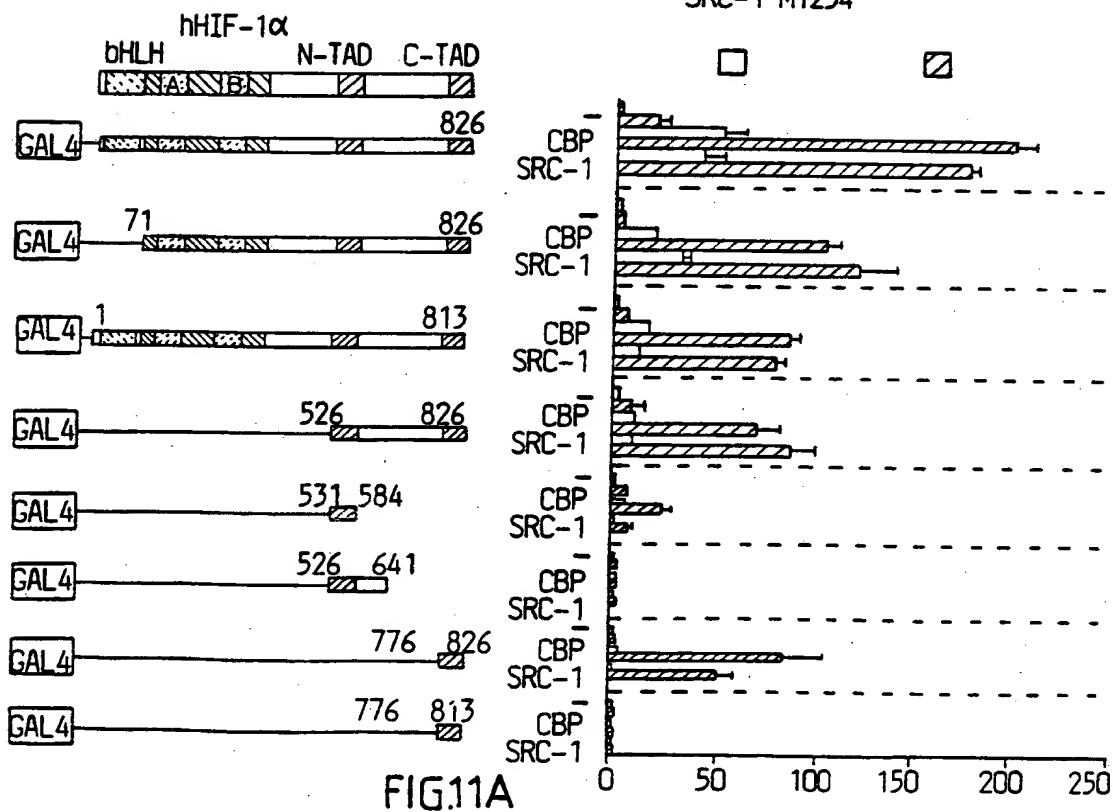
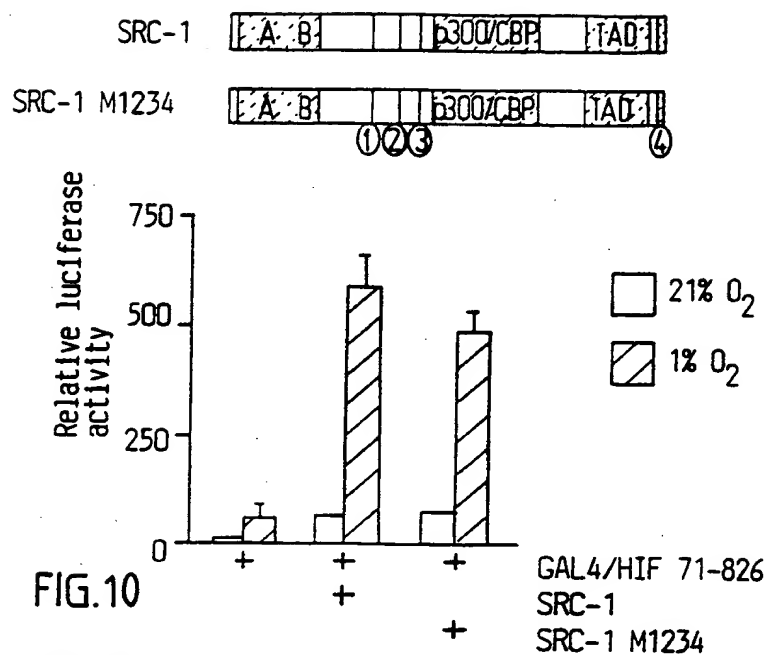


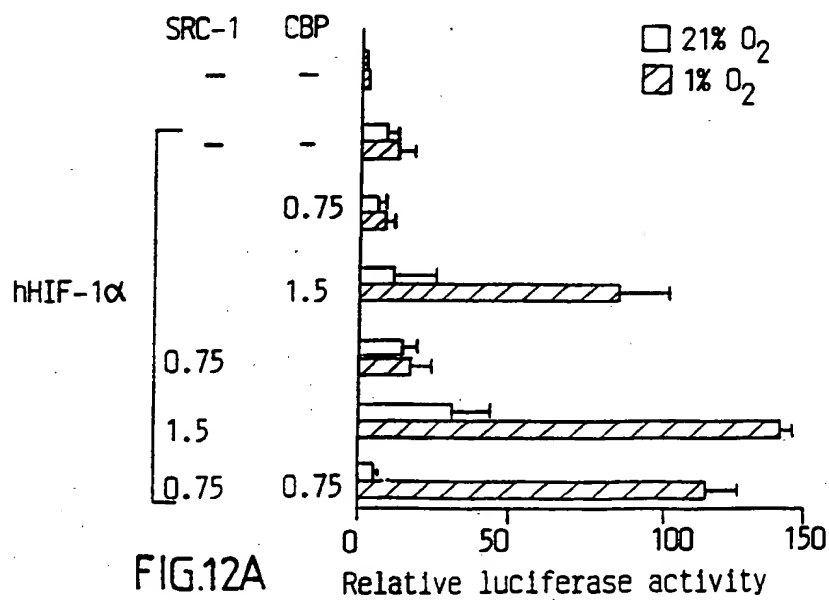
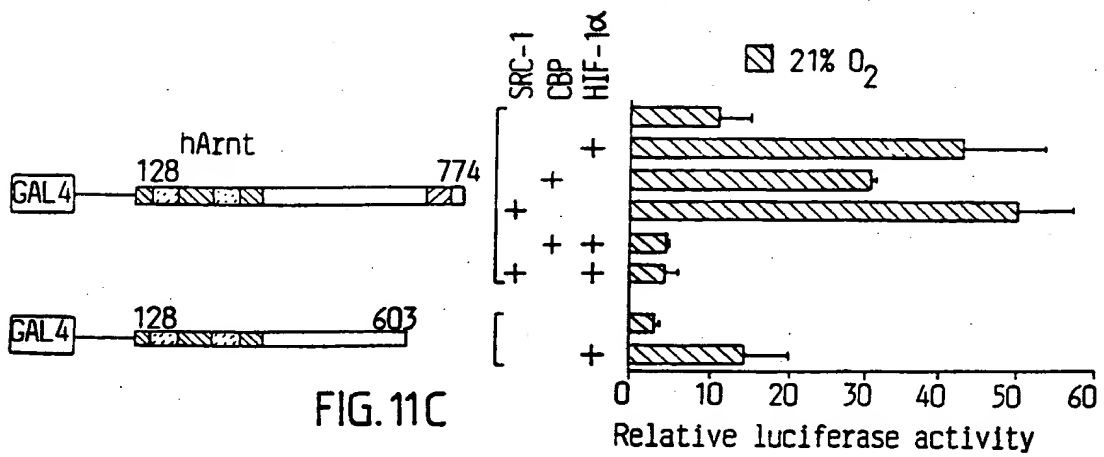
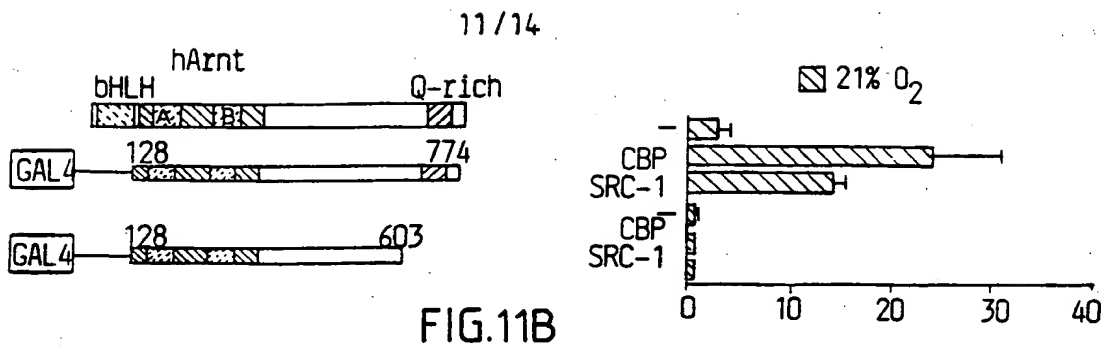
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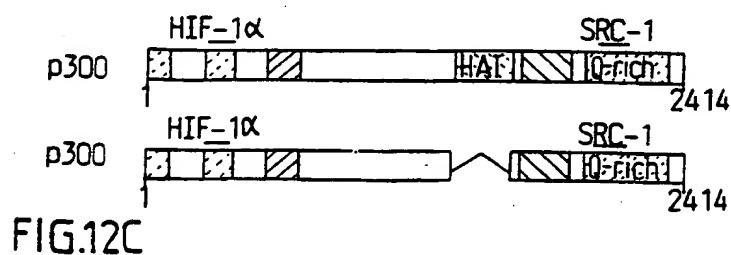
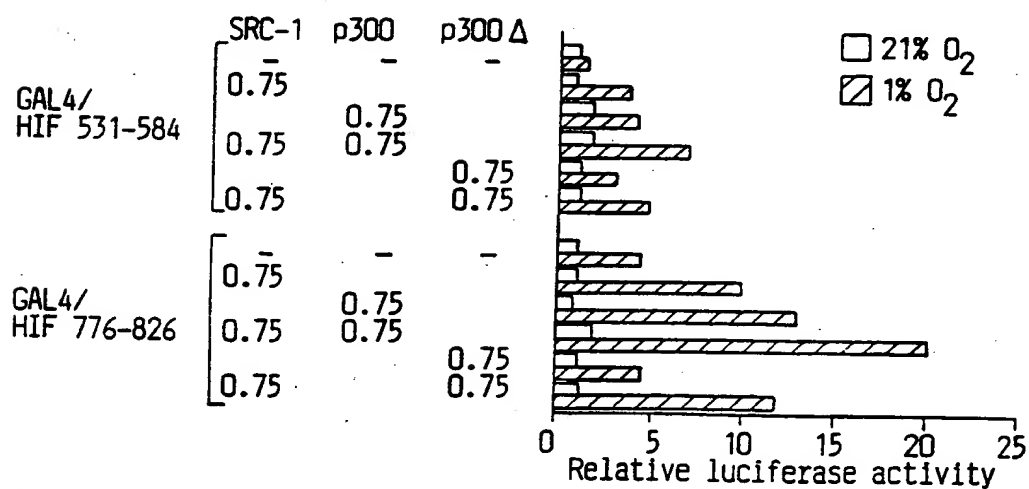
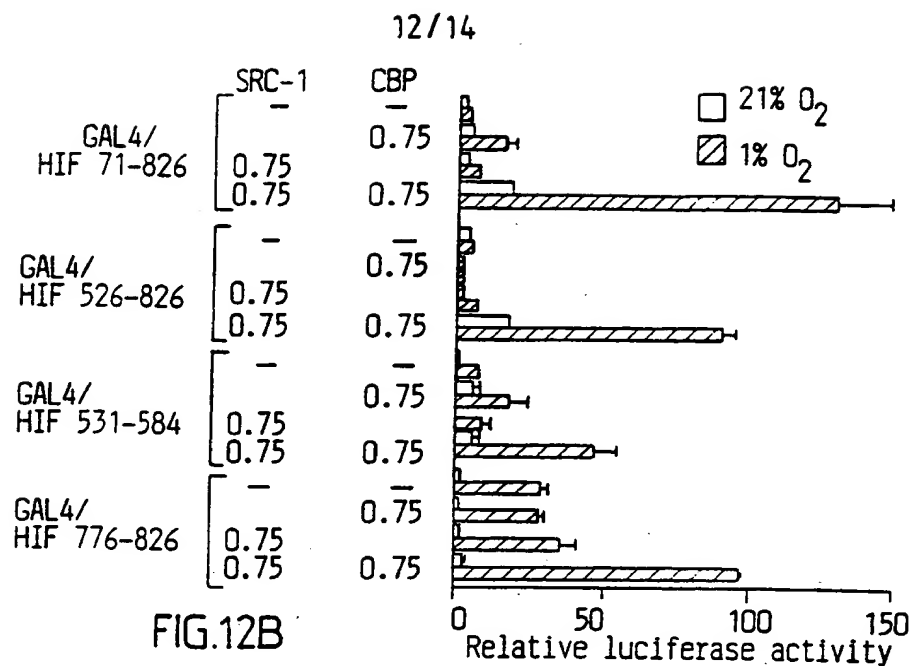




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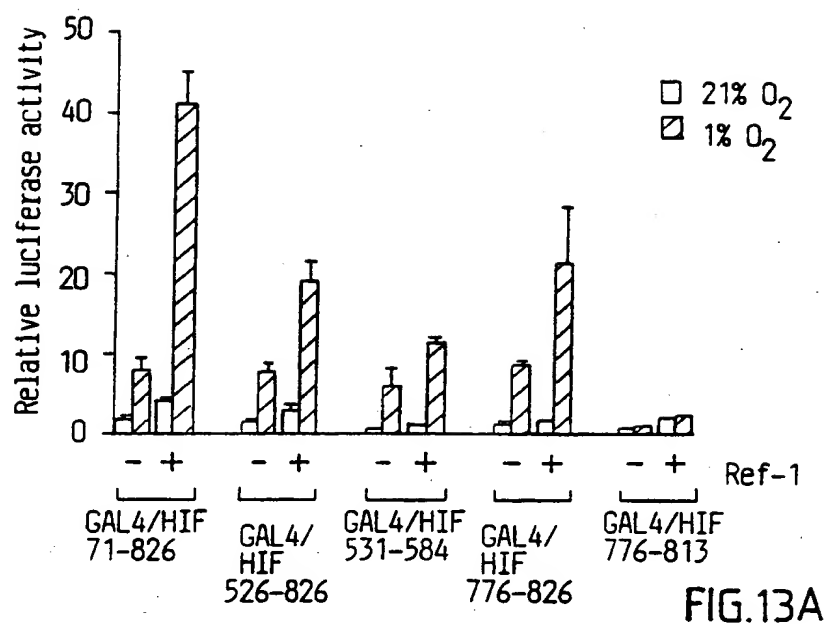


FIG.13A

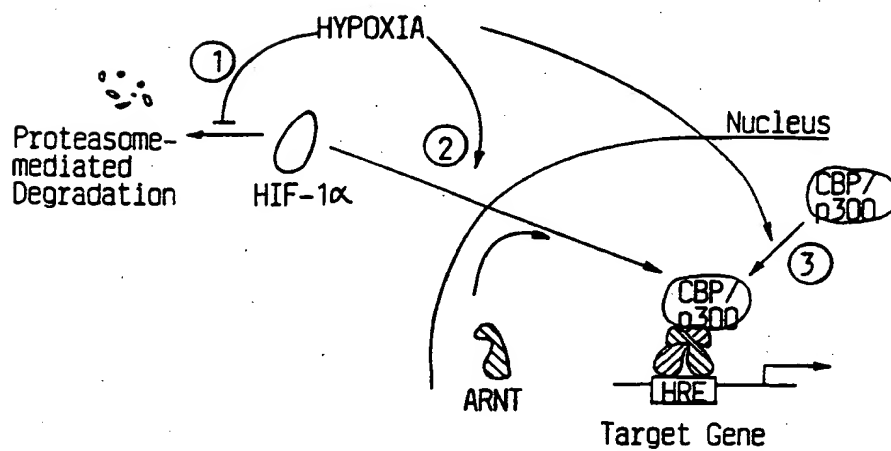


FIG.14

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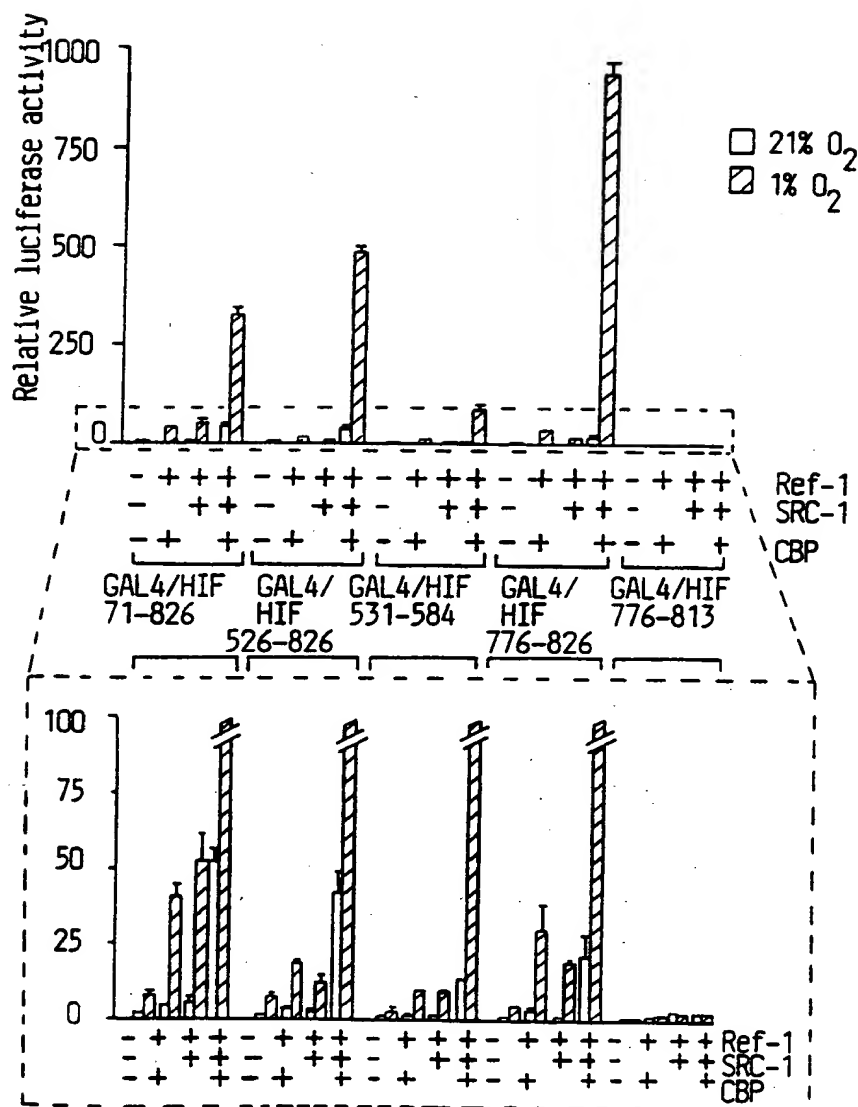


FIG.13B

SEQUENCE LISTING

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Asn Val Ser Ser His Leu Asp Lys Ala Ser Val Met Arg Leu Thr Ile
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Ser Tyr Leu Arg Val Arg Lys Leu Leu Asp Ala Gly Asp Leu Asp Ile
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 85 90 95

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Gln Asn Thr Gln Arg Ser Phe Phe Leu Arg Met Lys Cys Thr Leu Thr
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 Glu Ala Lys Asn Pro Phe Ser Thr Gln Asp Thr Asp Leu Asp Leu Glu
 545 550 555 560
 Met Leu Ala Pro Tyr Ile Pro Met Asp Asp Asp Phe Gln Leu Arg Ser
 565 570 575
 Phe Asp Gln Leu Ser Pro Leu Glu Ser Ser Ser Ala Ser Pro Glu Ser
 580 585 590
 Ala Ser Pro Gln Ser Thr Val Thr Val Phe Gln Gln Thr Gln Ile Gln
 595 600 605
 Glu Pro Thr Ala Asn Ala Thr Thr Thr Thr Ala Thr Thr Asp Glu Leu
 610 615 620
 Lys Thr Val Thr Lys Asp Arg Met Glu Asp Ile Lys Ile Leu Ile Ala
 625 630 635 640
 Ser Pro Ser Pro Thr His Ile His Lys Glu Thr Thr Ser Ala Thr Ser
 645 650 655
 Ser Pro Tyr Arg Asp Thr Gln Ser Arg Thr Ala Ser Pro Asn Arg Ala
 660 665 670
 Gly Lys Gly Val Ile Glu Gln Thr Glu Lys Ser His Pro Arg Ser Pro
 675 680 685
 Asn Val Leu Ser Val Ala Leu Ser Gln Arg Thr Thr Val Pro Glu Glu
 690 695 700
 Glu Leu Asn Pro Lys Ile Leu Ala Leu Gln Asn Ala Gln Arg Lys Arg
 705 710 715 720
 Lys Met Glu His Asp Gly Ser Leu Phe Gln Ala Val Gly Ile Gly Thr
 725 730 735
 Leu Leu Gln Gln Pro Asp Asp His Ala Ala Thr Thr Ser Leu Ser Trp
 740 745 750
 Lys Arg Val Lys Gly Cys Lys Ser Ser Glu Gln Asn Gly Met Glu Gln
 755 760 765
 Lys Thr Ile Ile Leu Ile Pro Ser Asp Leu Ala Cys Arg Leu Leu Gly
 770 775 780
 Gln Ser Met Asp Glu Ser Gly Leu Pro Gln Leu Thr Ser Tyr Asp Cys
 785 790 795 800
 Glu Val Asn Ala Pro Ile Gln Gly Ser Arg Asn Leu Leu Gln Gly Glu
 805 810 815
 Glu Leu Leu Arg Ala Leu Asp Gln Val Asn
 820 825

<210> 2

<211> 74
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Subdomain 1-74
 of human HIF-1 alpha

<400> 2
 Met Glu Gly Ala Gly Gly Ala Asn Asp Lys Lys Lys Ile Ser Ser Glu
 1 5 10 15
 Arg Arg Lys Glu Lys Ser Arg Asp Ala Ala Arg Ser Arg Arg Ser Lys
 20 25 30
 Glu Ser Glu Val Phe Tyr Glu Leu Ala His Gln Leu Pro Leu Pro His
 35 40 45
 Asn Val Ser Ser His Leu Asp Lys Ala Ser Val Met Arg Leu Thr Ile
 50 55 60
 Ser Tyr Leu Arg Val Arg Lys Leu Leu Asp
 65 70

<210> 3
 <211> 245
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Subdomain
 1-245 of human HIF-1 alpha

<400> 3
 Met Glu Gly Ala Gly Gly Ala Asn Asp Lys Lys Lys Ile Ser Ser Glu
 1 5 10 15
 Arg Arg Lys Glu Lys Ser Arg Asp Ala Ala Arg Ser Arg Arg Ser Lys
 20 25 30
 Glu Ser Glu Val Phe Tyr Glu Leu Ala His Gln Leu Pro Leu Pro His
 35 40 45
 Asn Val Ser Ser His Leu Asp Lys Ala Ser Val Met Arg Leu Thr Ile
 50 55 60
 Ser Tyr Leu Arg Val Arg Lys Leu Leu Asp Ala Gly Asp Leu Asp Ile
 65 70 75 80
 Glu Asp Asp Met Lys Ala Gln Met Asn Cys Phe Tyr Leu Lys Ala Leu
 85 90 95
 Asp Gly Phe Val Met Val Leu Thr Asp Asp Gly Asp Met Ile Tyr Ile
 100 105 110
 Ser Asp Asn Val Asn Lys Tyr Met Gly Leu Thr Gln Phe Glu Leu Thr
 115 120 125
 Gly His Ser Val Phe Asp Phe Thr His Pro Cys Asp His Glu Glu Met
 130 135 140

```

Arg Glu Met Leu Thr His Arg Asn Gly Leu Val Lys Lys Gly Lys Glu
145                      150                      155                      160

Gln Asn Thr Gln Arg Ser Phe Phe Leu Arg Met Lys Cys Thr Leu Thr
                      165                      170                      175

Ser Arg Gly Arg Thr Met Asn Ile Lys Ser Ala Thr Trp Lys Val Leu
                      180                      185                      190

His Cys Thr Gly His Ile His Val Tyr Asp Thr Asn Ser Asn Gln Pro
                      195                      200                      205

Gln Cys Gly Tyr Lys Lys Pro Pro Met Thr Cys Leu Val Leu Ile Cys
210                      215                      220

Glu Pro Ile Pro His Pro Ser Asn Ile Glu Ile Pro Leu Asp Ser Lys
225                      230                      235                      240

Thr Phe Leu Ser Arg
                      245

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```

<210> 4
<211> 330
<212> PRT
<213> Artificial Sequence

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<220>
<223> Description of Artificial Sequence: Subdomain
      1-330 of human HIF-1 alpha

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<400> 4
Met Glu Gly Ala Gly Gly Ala Asn Asp Lys Lys Lys Ile Ser Ser Glu
 1              5              10              15

Arg Arg Lys Glu Lys Ser Arg Asp Ala Ala Arg Ser Arg Arg Ser Lys
      20              25              30

Glu Ser Glu Val Phe Tyr Glu Leu Ala His Gln Leu Pro Leu Pro His
      35              40              45

Asn Val Ser Ser His Leu Asp Lys Ala Ser Val Met Arg Leu Thr Ile
      50              55              60

Ser Tyr Leu Arg Val Arg Lys Leu Leu Asp Ala Gly Asp Leu Asp Ile
      65              70              75              80

Glu Asp Asp Met Lys Ala Gln Met Asn Cys Phe Tyr Leu Lys Ala Leu
      85              90              95

Asp Gly Phe Val Met Val Leu Thr Asp Asp Gly Asp Met Ile Tyr Ile
      100             105             110

Ser Asp Asn Val Asn Lys Tyr Met Gly Leu Thr Gln Phe Glu Leu Thr
      115             120             125

Gly His Ser Val Phe Asp Phe Thr His Pro Cys Asp His Glu Glu Met
      130             135             140

Arg Glu Met Leu Thr His Arg Asn Gly Leu Val Lys Lys Gly Lys Glu
145                      150                      155                      160

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Gln Asn Thr Gln Arg Ser Phe Phe Leu Arg Met Lys Cys Thr Leu Thr
 165 170 175
 Ser Arg Gly Arg Thr Met Asn Ile Lys Ser Ala Thr Trp Lys Val Leu
 180 185 190
 His Cys Thr Gly His Ile His Val Tyr Asp Thr Asn Ser Asn Gln Pro
 195 200 205
 Gln Cys Gly Tyr Lys Lys Pro Pro Met Thr Cys Leu Val Leu Ile Cys
 210 215 220
 Glu Pro Ile Pro His Pro Ser Asn Ile Glu Ile Pro Leu Asp Ser Lys
 225 230 235 240
 Thr Phe Leu Ser Arg His Ser Leu Asp Met Lys Phe Ser Tyr Cys Asp
 245 250 255
 Glu Arg Ile Thr Glu Leu Met Gly Tyr Glu Pro Glu Glu Leu Leu Gly
 260 265 270
 Arg Ser Ile Tyr Glu Tyr Tyr His Ala Leu Asp Ser Asp His Leu Thr
 275 280 285
 Lys Thr His His Asp Met Phe Thr Lys Gly Gln Val Thr Thr Gly Gln
 290 295 300
 Tyr Arg Met Leu Ala Lys Arg Gly Gly Tyr Val Trp Val Glu Thr Gln
 305 310 315 320
 Ala Thr Val Ile Tyr Asn Thr Lys Asn Ser
 325 330

<210> 5
 <211> 652
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Subdomain
 1-652 of human HIF-1 alpha

<400> 5
 Met Glu Gly Ala Gly Gly Ala Asn Asp Lys Lys Lys Ile Ser Ser Glu
 1 5 10 15
 Arg Arg Lys Glu Lys Ser Arg Asp Ala Ala Arg Ser Arg Arg Ser Lys
 20 25 30
 Glu Ser Glu Val Phe Tyr Glu Leu Ala His Gln Leu Pro Leu Pro His
 35 40 45
 Asn Val Ser Ser His Leu Asp Lys Ala Ser Val Met Arg Leu Thr Ile
 50 55 60
 Ser Tyr Leu Arg Val Arg Lys Leu Leu Asp Ala Gly Asp Leu Asp Ile
 65 70 75 80
 Glu Asp Asp Met Lys Ala Gln Met Asn Cys Phe Tyr Leu Lys Ala Leu
 85 90 95

Asp Gly Phe Val Met Val Leu Thr Asp Asp Gly Asp Met Ile Tyr Ile
 100 105 110
 Ser Asp Asn Val Asn Lys Tyr Met Gly Leu Thr Gln Phe Glu Leu Thr
 115 120 125
 Gly His Ser Val Phe Asp Phe Thr His Pro Cys Asp His Glu Glu Met
 130 135 140
 Arg Glu Met Leu Thr His Arg Asn Gly Leu Val Lys Lys Gly Lys Glu
 145 150 155 160
 Gln Asn Thr Gln Arg Ser Phe Phe Leu Arg Met Lys Cys Thr Leu Thr
 165 170 175
 Ser Arg Gly Arg Thr Met Asn Ile Lys Ser Ala Thr Trp Lys Val Leu
 180 185 190
 His Cys Thr Gly His Ile His Val Tyr Asp Thr Asn Ser Asn Gln Pro
 195 200 205
 Gln Cys Gly Tyr Lys Lys Pro Pro Met Thr Cys Leu Val Leu Ile Cys
 210 215 220
 Glu Pro Ile Pro His Pro Ser Asn Ile Glu Ile Pro Leu Asp Ser Lys
 225 230 235 240
 Thr Phe Leu Ser Arg His Ser Leu Asp Met Lys Phe Ser Tyr Cys Asp
 245 250 255
 Glu Arg Ile Thr Glu Leu Met Gly Tyr Glu Pro Glu Glu Leu Leu Gly
 260 265 270
 Arg Ser Ile Tyr Glu Tyr Tyr His Ala Leu Asp Ser Asp His Leu Thr
 275 280 285
 Lys Thr His His Asp Met Phe Thr Lys Gly Gln Val Thr Thr Gly Gln
 290 295 300
 Tyr Arg Met Leu Ala Lys Arg Gly Gly Tyr Val Trp Val Glu Thr Gln
 305 310 315 320
 Ala Thr Val Ile Tyr Asn Thr Lys Asn Ser Gln Pro Gln Cys Ile Val
 325 330 335
 Cys Val Asn Tyr Val Val Ser Gly Ile Ile Gln His Asp Leu Ile Phe
 340 345 350
 Ser Leu Gln Thr Glu Cys Val Leu Lys Pro Val Glu Ser Ser Asp
 355 360 365
 Met Lys Met Thr Gln Leu Phe Thr Lys Val Glu Ser Glu Asp Thr Ser
 370 375 380
 Ser Leu Phe Asp Lys Leu Lys Lys Glu Pro Asp Ala Leu Thr Leu Leu
 385 390 395 400
 Ala Pro Ala Ala Gly Asp Thr Ile Ile Ser Leu Asp Phe Gly Ser Asn
 405 410 415
 Asp Thr Glu Thr Asp Asp Gln Gln Leu Glu Glu Val Pro Leu Tyr Asn
 420 425 430

Asp Val Met Leu Pro Ser Pro Asn Glu Lys Leu Gln Asn Ile Asn Leu
 435 440 445
 Ala Met Ser Pro Leu Pro Thr Ala Glu Thr Pro Lys Pro Leu Arg Ser
 450 455 460
 Ser Ala Asp Pro Ala Leu Asn Gln Glu Val Ala Leu Lys Leu Glu Pro
 465 470 475 480
 Asn Pro Glu Ser Leu Glu Leu Ser Phe Thr Met Pro Gln Ile Gln Asp
 485 490 495
 Gln Thr Pro Ser Pro Ser Asp Gly Ser Thr Arg Gln Ser Ser Pro Glu
 500 505 510
 Pro Asn Ser Pro Ser Glu Tyr Cys Phe Tyr Val Asp Ser Asp Met Val
 515 520 525
 Asn Glu Phe Lys Leu Glu Leu Val Glu Lys Leu Phe Ala Glu Asp Thr
 530 535 540
 Glu Ala Lys Asn Pro Phe Ser Thr Gln Asp Thr Asp Leu Asp Leu Glu
 545 550 555 560
 Met Leu Ala Pro Tyr Ile Pro Met Asp Asp Asp Phe Gln Leu Arg Ser
 565 570 575
 Phe Asp Gln Leu Ser Pro Leu Glu Ser Ser Ser Ala Ser Pro Glu Ser
 580 585 590
 Ala Ser Pro Gln Ser Thr Val Thr Val Phe Gln Gln Thr Gln Ile Gln
 595 600 605
 Glu Pro Thr Ala Asn Ala Thr Thr Thr Thr Ala Thr Thr Asp Glu Leu
 610 615 620
 Lys Thr Val Thr Lys Asp Arg Met Glu Asp Ile Lys Ile Leu Ile Ala
 625 630 635 640
 Ser Pro Ser Pro Thr His Ile His Lys Glu Thr Thr
 645 650

<210> 6

<211> 613

<212> PRT

<213> Artificial Sequence

<400> 6

Met Glu Gly Ala Gly Gly Ala Asn Asp Lys Lys Lys Ile Ser Ser Glu
 1 5 10 15
 Arg Arg Lys Glu Lys Ser Arg Asp Ala Arg Ser Arg Arg Ser Lys
 20 25 30
 Glu Ser Glu Val Phe Tyr Glu Leu Ala His Gln Leu Pro Leu Pro His
 35 40 45
 Asn Val Ser Ser His Leu Asp Lys Ala Ser Val Met Arg Leu Thr Ile
 50 55 60

Ser Tyr Leu Arg Val Arg Lys Leu Leu Asp Ala Gly Asp Leu Asp Ile
 65 70 75 80
 Glu Asp Asp Met Lys Ala Gln Met Asn Cys Phe Tyr Leu Lys Ala Leu
 85 90 95
 Asp Gly Phe Val Met Val Leu Thr Asp Asp Gly Asp Met Ile Tyr Ile
 100 105 110
 Ser Asp Asn Val Asn Lys Tyr Met Gly Leu Thr Gln Phe Glu Leu Thr
 115 120 125
 Gly His Ser Val Phe Asp Phe Thr His Pro Cys Asp His Glu Glu Met
 130 135 140
 Arg Glu Met Leu Thr His Arg Asn Gly Leu Val Lys Lys Gly Lys Glu
 145 150 155 160
 Gln Asn Thr Gln Arg Ser Phe Phe Leu Arg Met Lys Cys Thr Leu Thr
 165 170 175
 Ser Lys Lys Glu Pro Asp Ala Leu Thr Leu Leu Ala Pro Ala Ala Gly
 180 185 190
 Asp Thr Ile Ile Ser Leu Asp Phe Gly Ser Asn Asp Thr Glu Thr Asp
 195 200 205
 Asp Gln Gln Leu Glu Glu Val Pro Leu Tyr Asn Asp Val Met Leu Pro
 210 215 220
 Ser Pro Asn Glu Lys Leu Gln Asn Ile Asn Leu Ala Met Ser Pro Leu
 225 230 235 240
 Pro Thr Ala Glu Thr Pro Lys Pro Leu Arg Ser Ser Ala Asp Pro Ala
 245 250 255
 Leu Asn Gln Glu Val Ala Leu Lys Leu Glu Pro Asn Pro Glu Ser Leu
 260 265 270
 Glu Leu Ser Phe Thr Met Pro Gln Ile Gln Asp Gln Thr Pro Ser Pro
 275 280 285
 Ser Asp Gly Ser Thr Arg Gln Ser Ser Pro Glu Pro Asn Ser Pro Ser
 290 295 300
 Glu Tyr Cys Phe Tyr Val Asp Ser Asp Met Val Asn Glu Phe Lys Leu
 305 310 315 320
 Glu Leu Val Glu Lys Leu Phe Ala Glu Asp Thr Glu Ala Lys Asn Pro
 325 330 335
 Phe Ser Thr Gln Asp Thr Asp Leu Asp Leu Glu Met Leu Ala Pro Tyr
 340 345 350
 Ile Pro Met Asp Asp Asp Phe Gln Leu Arg Ser Phe Asp Gln Leu Ser
 355 360 365
 Pro Leu Glu Ser Ser Ser Ala Ser Pro Glu Ser Ala Ser Pro Gln Ser
 370 375 380
 Thr Val Thr Val Phe Gln Gln Thr Gln Ile Gln Glu Pro Thr Ala Asn
 385 390 395 400

Ala Thr Thr Thr Thr Ala Thr Thr Asp Glu Leu Lys Thr Val Thr Lys
 405 410 415
 Asp Arg Met Glu Asp Ile Lys Ile Leu Ile Ala Ser Pro Ser Pro Thr
 420 425 430
 His Ile His Lys Glu Thr Thr Ser Ala Thr Ser Ser Pro Tyr Arg Asp
 435 440 445
 Thr Gln Ser Arg Thr Ala Ser Pro Asn Arg Ala Gly Lys Gly Val Ile
 450 455 460
 Glu Gln Thr Glu Lys Ser His Pro Arg Ser Pro Asn Val Leu Ser Val
 465 470 475 480
 Ala Leu Ser Gln Arg Thr Thr Val Pro Glu Glu Glu Leu Asn Pro Lys
 485 490 495
 Ile Leu Ala Leu Gln Asn Ala Gln Arg Lys Arg Lys Met Glu His Asp
 500 505 510
 Gly Ser Leu Phe Gln Ala Val Gly Ile Gly Thr Leu Leu Gln Gln Pro
 515 520 525
 Asp Asp His Ala Ala Thr Thr Ser Leu Ser Trp Lys Arg Val Lys Gly
 530 535 540
 Cys Lys Ser Ser Glu Gln Asn Gly Met Glu Gln Lys Thr Ile Ile Leu
 545 550 555 560
 Ile Pro Ser Asp Leu Ala Cys Arg Leu Leu Gly Gln Ser Met Asp Glu
 565 570 575
 Ser Gly Leu Pro Gln Leu Thr Ser Tyr Asp Cys Glu Val Asn Ala Pro
 580 585 590
 Ile Gln Gly Ser Arg Asn Leu Leu Gln Gly Glu Glu Leu Leu Arg Ala
 595 600 605
 Leu Asp Gln Val Asn
 610

<210> 7

<211> 311

<212> PRT

<213> Artificial Sequence

<220>

 <223> Description of Artificial Sequence: Subdomain
 331-641 of human HIF-1 alpha

<400> 7

 Gln Pro Gln Cys Ile Val Cys Val Asn Tyr Val Val Ser Gly Ile Ile
 1 5 10 15

 Gln His Asp Leu Ile Phe Ser Leu Gln Gln Thr Glu Cys Val Leu Lys
 20 25 30

 Pro Val Glu Ser Ser Asp Met Lys Met Thr Gln Leu Phe Thr Lys Val
 35 40 45

<400> 8

12

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Asp Met Val Asn Glu Phe Lys Leu Glu Leu Val Glu Lys Leu Phe Ala
 1           5           10           15
Glu Asp Thr Glu Ala Lys Asn Pro Phe Ser Thr Gln Asp Thr Asp Leu
          20           25           30
Asp Leu Glu Met Leu Ala Pro Tyr Ile Pro Met Asp Asp Asp Phe Gln
          35           40           45
Leu Arg Ser Phe Asp Gln Leu Ser Pro Leu Glu Ser Ser Ser Ala Ser
          50           55           60
Pro Glu Ser Ala Ser Pro Gln Ser Thr Val Thr Val Phe Gln Gln Thr
          65           70           75           80
Gln Ile Gln Glu Pro Thr Ala Asn Ala Thr Thr Thr Thr Ala Thr Thr
          85           90           95
Asp Glu Leu Lys Thr Val Thr Lys Asp Arg Met Glu Asp Ile Lys Ile
          100          105          110
Leu Ile Ala Ser
          115

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<210> 9

<211> 288

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Subdomain
526-813 of human HIF-1 alpha

<400> 9

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Asp Met Val Asn Glu Phe Lys Leu Glu Leu Val Glu Lys Leu Phe Ala
 1           5           10           15
Glu Asp Thr Glu Ala Lys Asn Pro Phe Ser Thr Gln Asp Thr Asp Leu
          20           25           30
Asp Leu Glu Met Leu Ala Pro Tyr Ile Pro Met Asp Asp Asp Phe Gln
          35           40           45
Leu Arg Ser Phe Asp Gln Leu Ser Pro Leu Glu Ser Ser Ser Ala Ser
          50           55           60
Pro Glu Ser Ala Ser Pro Gln Ser Thr Val Thr Val Phe Gln Gln Thr
          65           70           75           80
Gln Ile Gln Glu Pro Thr Ala Asn Ala Thr Thr Thr Thr Ala Thr Thr
          85           90           95
Asp Glu Leu Lys Thr Val Thr Lys Asp Arg Met Glu Asp Ile Lys Ile
          100          105          110
Leu Ile Ala Ser Pro Ser Pro Thr His Ile His Lys Glu Thr Thr Ser
          115          120          125
Ala Thr Ser Ser Pro Tyr Arg Asp Thr Gln Ser Arg Thr Ala Ser Pro
          130          135          140

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Asn Arg Ala Gly Lys Gly Val Ile Glu Gln Thr Glu Lys Ser His Pro
145                150                155                160

Arg Ser Pro Asn Val Leu Ser Val Ala Leu Ser Gln Arg Thr Thr Val
                165                170                175

Pro Glu Glu Glu Leu Asn Pro Lys Ile Leu Ala Leu Gln Asn Ala Gln
                180                185                190

Arg Lys Arg Lys Met Glu His Asp Gly Ser Leu Phe Gln Ala Val Gly
195                200                205

Ile Gly Thr Leu Leu Gln Gln Pro Asp Asp His Ala Ala Thr Thr Ser
210                215                220

Leu Ser Trp Lys Arg Val Lys Gly Cys Lys Ser Ser Glu Gln Asn Gly
225                230                235                240

Met Glu Gln Lys Thr Ile Ile Leu Ile Pro Ser Asp Leu Ala Cys Arg
245                250                255

Leu Leu Gly Gln Ser Met Asp Glu Ser Gly Leu Pro Gln Leu Thr Ser
260                265                270

Tyr Asp Cys Glu Val Asn Ala Pro Ile Gln Gly Ser Arg Asn Leu Leu
275                280                285

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<210> 10
<211> 301
<212> PRT
<213> Artificial Sequence

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<220>
<223> Description of Artificial Sequence: Subdomain
526-826 of human HIF-1 alpha

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```

<400> 10
Asp Met Val Asn Glu Phe Lys Leu Glu Leu Val Glu Lys Leu Phe Ala
1                5                10                15

Glu Asp Thr Glu Ala Lys Asn Pro Phe Ser Thr Gln Asp Thr Asp Leu
20                25                30

Asp Leu Glu Met Leu Ala Pro Tyr Ile Pro Met Asp Asp Asp Phe Gln
35                40                45

Leu Arg Ser Phe Asp Gln Leu Ser Pro Leu Glu Ser Ser Ser Ala Ser
50                55                60

Pro Glu Ser Ala Ser Pro Gln Ser Thr Val Thr Val Phe Gln Gln Thr
65                70                75                80

Gln Ile Gln Glu Pro Thr Ala Asn Ala Thr Thr Thr Thr Ala Thr Thr
85                90                95

Asp Glu Leu Lys Thr Val Thr Lys Asp Arg Met Glu Asp Ile Lys Ile
100               105               110

```

Leu Ile Ala Ser Pro Ser Pro Thr His Ile His Lys Glu Thr Thr Ser
 115 120 125
 Ala Thr Ser Ser Pro Tyr Arg Asp Thr Gln Ser Arg Thr Ala Ser Pro
 130 135 140
 Asn Arg Ala Gly Lys Gly Val Ile Glu Gln Thr Glu Lys Ser His Pro
 145 150 155 160
 Arg Ser Pro Asn Val Leu Ser Val Ala Leu Ser Gln Arg Thr Thr Val
 165 170 175
 Pro Glu Glu Glu Leu Asn Pro Lys Ile Leu Ala Leu Gln Asn Ala Gln
 180 185 190
 Arg Lys Arg Lys Met Glu His Asp Gly Ser Leu Phe Gln Ala Val Gly
 195 200 205
 Ile Gly Thr Leu Leu Gln Gln Pro Asp Asp His Ala Ala Thr Thr Ser
 210 215 220
 Leu Ser Trp Lys Arg Val Lys Gly Cys Lys Ser Ser Glu Gln Asn Gly
 225 230 235 240
 Met Glu Gln Lys Thr Ile Ile Leu Ile Pro Ser Asp Leu Ala Cys Arg
 245 250 255
 Leu Leu Gly Gln Ser Met Asp Glu Ser Gly Leu Pro Gln Leu Thr Ser
 260 265 270
 Tyr Asp Cys Glu Val Asn Ala Pro Ile Gln Gly Ser Arg Asn Leu Leu
 275 280 285
 Gln Gly Glu Glu Leu Leu Arg Ala Leu Asp Gln Val Asn
 290 295 300

<210> 11
 <211> 756
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Subdomain
 71-826 of human HIF-1 alpha

<400> 11
 Lys Leu Leu Asp Ala Gly Asp Leu Asp Ile Glu Asp Asp Met Lys Ala
 1 5 10 15
 Gln Met Asn Cys Phe Tyr Leu Lys Ala Leu Asp Gly Phe Val Met Val
 20 25 30
 Leu Thr Asp Asp Gly Asp Met Ile Tyr Ile Ser Asp Asn Val Asn Lys
 35 40 45
 Tyr Met Gly Leu Thr Gln Phe Glu Leu Thr Gly His Ser Val Phe Asp
 50 55 60
 Phe Thr His Pro Cys Asp His Glu Glu Met Arg Glu Met Leu Thr His
 65 70 75 80

Arg Asn Gly Leu Val Lys Lys Gly Lys Glu Gln Asn Thr Gln Arg Ser
 85 90 95
 Phe Phe Leu Arg Met Lys Cys Thr Leu Thr Ser Arg Gly Arg Thr Met
 100 105 110
 Asn Ile Lys Ser Ala Thr Trp Lys Val Leu His Cys Thr Gly His Ile
 115 120 125
 His Val Tyr Asp Thr Asn Ser Asn Gln Pro Gln Cys Gly Tyr Lys Lys
 130 135 140
 Pro Pro Met Thr Cys Leu Val Leu Ile Cys Glu Pro Ile Pro His Pro
 145 150 155 160
 Ser Asn Ile Glu Ile Pro Leu Asp Ser Lys Thr Phe Leu Ser Arg His
 165 170 175
 Ser Leu Asp Met Lys Phe Ser Tyr Cys Asp Glu Arg Ile Thr Glu Leu
 180 185 190
 Met Gly Tyr Glu Pro Glu Glu Leu Leu Gly Arg Ser Ile Tyr Glu Tyr
 195 200 205
 Tyr His Ala Leu Asp Ser Asp His Leu Thr Lys Thr His His Asp Met
 210 215 220
 Phe Thr Lys Gly Gln Val Thr Thr Gly Gln Tyr Arg Met Leu Ala Lys
 225 230 235 240
 Arg Gly Gly Tyr Val Trp Val Glu Thr Gln Ala Thr Val Ile Tyr Asn
 245 250 255
 Thr Lys Asn Ser Gln Pro Gln Cys Ile Val Cys Val Asn Tyr Val Val
 260 265 270
 Ser Gly Ile Ile Gln His Asp Leu Ile Phe Ser Leu Gln Gln Thr Glu
 275 280 285
 Cys Val Leu Lys Pro Val Glu Ser Ser Asp Met Lys Met Thr Gln Leu
 290 295 300
 Phe Thr Lys Val Glu Ser Glu Asp Thr Ser Ser Leu Phe Asp Lys Leu
 305 310 315 320
 Lys Lys Glu Pro Asp Ala Leu Thr Leu Leu Ala Pro Ala Ala Gly Asp
 325 330 335
 Thr Ile Ile Ser Leu Asp Phe Gly Ser Asn Asp Thr Glu Thr Asp Asp
 340 345 350
 Gln Gln Leu Glu Glu Val Pro Leu Tyr Asn Asp Val Met Leu Pro Ser
 355 360 365
 Pro Asn Glu Lys Leu Gln Asn Ile Asn Leu Ala Met Ser Pro Leu Pro
 370 375 380
 Thr Ala Glu Thr Pro Lys Pro Leu Arg Ser Ser Ala Asp Pro Ala Leu
 385 390 395 400
 Asn Gln Glu Val Ala Leu Lys Leu Glu Pro Asn Pro Glu Ser Leu Glu
 405 410 415

Leu Ser Phe Thr Met Pro Gln Ile Gln Asp Gln Thr Pro Ser Pro Ser
 420 425 430
 Asp Gly Ser Thr Arg Gln Ser Ser Pro Glu Pro Asn Ser Pro Ser Glu
 435 440 445
 Tyr Cys Phe Tyr Val Asp Ser Asp Met Val Asn Glu Phe Lys Leu Glu
 450 455 460
 Leu Val Glu Lys Leu Phe Ala Glu Asp Thr Glu Ala Lys Asn Pro Phe
 465 470 475 480
 Ser Thr Gln Asp Thr Asp Leu Asp Leu Glu Met Leu Ala Pro Tyr Ile
 485 490 495
 Pro Met Asp Asp Asp Phe Gln Leu Arg Ser Phe Asp Gln Leu Ser Pro
 500 505 510
 Leu Glu Ser Ser Ser Ala Ser Pro Glu Ser Ala Ser Pro Gln Ser Thr
 515 520 525
 Val Thr Val Phe Gln Gln Thr Gln Ile Gln Glu Pro Thr Ala Asn Ala
 530 535 540
 Thr Thr Thr Thr Ala Thr Thr Asp Glu Leu Lys Thr Val Thr Lys Asp
 545 550 555 560
 Arg Met Glu Asp Ile Lys Ile Leu Ile Ala Ser Pro Ser Pro Thr His
 565 570 575
 Ile His Lys Glu Thr Thr Ser Ala Thr Ser Ser Pro Tyr Arg Asp Thr
 580 585 590
 Gln Ser Arg Thr Ala Ser Pro Asn Arg Ala Gly Lys Gly Val Ile Glu
 595 600 605
 Gln Thr Glu Lys Ser His Pro Arg Ser Pro Asn Val Leu Ser Val Ala
 610 615 620
 Leu Ser Gln Arg Thr Thr Val Pro Glu Glu Glu Leu Asn Pro Lys Ile
 625 630 635 640
 Leu Ala Leu Gln Asn Ala Gln Arg Lys Arg Lys Met Glu His Asp Gly
 645 650 655
 Ser Leu Phe Gln Ala Val Gly Ile Gly Thr Leu Leu Gln Gln Pro Asp
 660 665 670
 Asp His Ala Ala Thr Thr Ser Leu Ser Trp Lys Arg Val Lys Gly Cys
 675 680 685
 Lys Ser Ser Glu Gln Asn Gly Met Glu Gln Lys Thr Ile Ile Leu Ile
 690 695 700
 Pro Ser Asp Leu Ala Cys Arg Leu Leu Gly Gln Ser Met Asp Glu Ser
 705 710 715 720
 Gly Leu Pro Gln Leu Thr Ser Tyr Asp Cys Glu Val Asn Ala Pro Ile
 725 730 735
 Gln Gly Ser Arg Asn Leu Leu Gln Gly Glu Glu Leu Leu Arg Ala Leu
 740 745 750

Asp Gln Val Asn
755

<210> 12
<211> 813
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Subdomain
1-813 of human HIF-1 alpha

<400> 12
Met Glu Gly Ala Gly Gly Ala Asn Asp Lys Lys Lys Ile Ser Ser Glu
1 5 10 15
Arg Arg Lys Glu Lys Ser Arg Asp Ala Ala Arg Ser Arg Arg Ser Lys
20 25 30
Glu Ser Glu Val Phe Tyr Glu Leu Ala His Gln Leu Pro Leu Pro His
35 40 45
Asn Val Ser Ser His Leu Asp Lys Ala Ser Val Met Arg Leu Thr Ile
50 55 60
Ser Tyr Leu Arg Val Arg Lys Leu Leu Asp Ala Gly Asp Leu Asp Ile
65 70 75 80
Glu Asp Asp Met Lys Ala Gln Met Asn Cys Phe Tyr Leu Lys Ala Leu
85 90 95
Asp Gly Phe Val Met Val Leu Thr Asp Asp Gly Asp Met Ile Tyr Ile
100 105 110
Ser Asp Asn Val Asn Lys Tyr Met Gly Leu Thr Gln Phe Glu Leu Thr
115 120 125
Gly His Ser Val Phe Asp Phe Thr His Pro Cys Asp His Glu Glu Met
130 135 140
Arg Glu Met Leu Thr His Arg Asn Gly Leu Val Lys Lys Gly Lys Glu
145 150 155 160
Gln Asn Thr Gln Arg Ser Phe Phe Leu Arg Met Lys Cys Thr Leu Thr
165 170 175
Ser Arg Gly Arg Thr Met Asn Ile Lys Ser Ala Thr Trp Lys Val Leu
180 185 190
His Cys Thr Gly His Ile His Val Tyr Asp Thr Asn Ser Asn Gln Pro
195 200 205
Gln Cys Gly Tyr Lys Lys Pro Pro Met Thr Cys Leu Val Leu Ile Cys
210 215 220
Glu Pro Ile Pro His Pro Ser Asn Ile Glu Ile Pro Leu Asp Ser Lys
225 230 235 240
Thr Phe Leu Ser Arg His Ser Leu Asp Met Lys Phe Ser Tyr Cys Asp
245 250 255

Glu Arg Ile Thr Glu Leu Met Gly Tyr Glu Pro Glu Glu Leu Leu Gly
 260 265 270
 Arg Ser Ile Tyr Glu Tyr Tyr His Ala Leu Asp Ser Asp His Leu Thr
 275 280 285
 Lys Thr His His Asp Met Phe Thr Lys Gly Gln Val Thr Thr Gly Gln
 290 295 300
 Tyr Arg Met Leu Ala Lys Arg Gly Gly Tyr Val Trp Val Glu Thr Gln
 305 310 315 320
 Ala Thr Val Ile Tyr Asn Thr Lys Asn Ser Gln Pro Gln Cys Ile Val
 325 330 335
 Cys Val Asn Tyr Val Val Ser Gly Ile Ile Gln His Asp Leu Ile Phe
 340 345 350
 Ser Leu Gln Gln Thr Glu Cys Val Leu Lys Pro Val Glu Ser Ser Asp
 355 360 365
 Met Lys Met Thr Gln Leu Phe Thr Lys Val Glu Ser Glu Asp Thr Ser
 370 375 380
 Ser Leu Phe Asp Lys Leu Lys Lys Glu Pro Asp Ala Leu Thr Leu Leu
 385 390 395 400
 Ala Pro Ala Ala Gly Asp Thr Ile Ile Ser Leu Asp Phe Gly Ser Asn
 405 410 415
 Asp Thr Glu Thr Asp Asp Gln Gln Leu Glu Glu Val Pro Leu Tyr Asn
 420 425 430
 Asp Val Met Leu Pro Ser Pro Asn Glu Lys Leu Gln Asn Ile Asn Leu
 435 440 445
 Ala Met Ser Pro Leu Pro Thr Ala Glu Thr Pro Lys Pro Leu Arg Ser
 450 455 460
 Ser Ala Asp Pro Ala Leu Asn Gln Glu Val Ala Leu Lys Leu Glu Pro
 465 470 475 480
 Asn Pro Glu Ser Leu Glu Leu Ser Phe Thr Met Pro Gln Ile Gln Asp
 485 490 495
 Gln Thr Pro Ser Pro Ser Asp Gly Ser Thr Arg Gln Ser Ser Pro Glu
 500 505 510
 Pro Asn Ser Pro Ser Glu Tyr Cys Phe Tyr Val Asp Ser Asp Met Val
 515 520 525
 Asn Glu Phe Lys Leu Glu Leu Val Glu Lys Leu Phe Ala Glu Asp Thr
 530 535 540
 Glu Ala Lys Asn Pro Phe Ser Thr Gln Asp Thr Asp Leu Asp Leu Glu
 545 550 555 560
 Met Leu Ala Pro Tyr Ile Pro Met Asp Asp Asp Phe Gln Leu Arg Ser
 565 570 575
 Phe Asp Gln Leu Ser Pro Leu Glu Ser Ser Ser Ala Ser Pro Glu Ser
 580 585 590

Ala Ser Pro Gln Ser Thr Val Thr Val Phe Gln Gln Thr Gln Ile Gln
595 600 605

Glu Pro Thr Ala Asn Ala Thr Thr Thr Thr Ala Thr Thr Asp Glu Leu
610 615 620

Lys Thr Val Thr Lys Asp Arg Met Glu Asp Ile Lys Ile Leu Ile Ala
625 630 635 640

Ser Pro Ser Pro Thr His Ile His Lys Glu Thr Thr Ser Ala Thr Ser
645 650 655

Ser Pro Tyr Arg Asp Thr Gln Ser Arg Thr Ala Ser Pro Asn Arg Ala
660 665 670

Gly Lys Gly Val Ile Glu Gln Thr Glu Lys Ser His Pro Arg Ser Pro
675 680 685

Asn Val Leu Ser Val Ala Leu Ser Gln Arg Thr Thr Val Pro Glu Glu
690 695 700

Glu Leu Asn Pro Lys Ile Leu Ala Leu Gln Asn Ala Gln Arg Lys Arg
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Lys Met Glu His Asp Gly Ser Leu Phe Gln Ala Val Gly Ile Gly Thr
725 730 735

Leu Leu Gln Gln Pro Asp Asp His Ala Ala Thr Thr Ser Leu Ser Trp
740 745 750

Lys Arg Val Lys Gly Cys Lys Ser Ser Glu Gln Asn Gly Met Glu Gln
755 760 765

Lys Thr Ile Ile Leu Ile Pro Ser Asp Leu Ala Cys Arg Leu Leu Gly
770 775 780

Gln Ser Met Asp Glu Ser Gly Leu Pro Gln Leu Thr Ser Tyr Asp Cys
785 790 795 800

Glu Val Asn Ala Pro Ile Gln Gly Ser Arg Asn Leu Leu
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<211> 54

<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Subdomain
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<400> 13

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Lys Asn Pro Phe Ser Thr Gln Asp Thr Asp Leu Asp Leu Glu Met Leu
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Ala Pro Tyr Ile Pro Met Asp Asp Asp Phe Gln Leu Arg Ser Phe Asp
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Gln Leu Ser Pro Leu Glu
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<213> Artificial Sequence

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Leu Pro Gln Leu Thr Ser Tyr Asp Cys Glu Val Asn Ala Pro Ile Gln
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Gly Ser Arg Asn Leu Leu Gln Gly Glu Glu Leu Leu Arg Ala Leu Asp
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Gln Val Asn
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<210> 15
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Subdomain
776-813 of human HIF-1 alpha

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1 5 10 15
Leu Pro Gln Leu Thr Ser Tyr Asp Cys Glu Val Asn Ala Pro Ile Gln
20 25 30
Gly Ser Arg Asn Leu Leu
35

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 99/02053

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 14/47, C12N 15/12, C12N 5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JBC Online, "Transactivation and Inhibitory Domains of Hypoxia-inducible Factor 1" Volume 272, Number 31, Issue of August 1, 1977, Bing-Hua Jiang et al http://www.jbc.org/cgi/content/full/272/31/19253	1,5-12,14, 16,18
A	--	2-4,13,15, 17,19-20
A	WO 9639426 A1 (THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE), 12 December 1996 (12.12.96) --	1-20

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

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"P" document published prior to the international filing date but later than the priority date claimed

"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

2 March 2000

Date of mailing of the international search report

09-03-2000

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 99/02053

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>Molecular and Cellular Biology, "Regulation of Transcription by Hypoxia Requires a Multiprotein Complex That Includes Hypoxia-Inducible Factor 1, an Adjacent Transcription Factor, and p300/CREB Binding Protein" Vol 18, No 7, July 1998 Benjamin L. Ebert et al http://mcb.asm.org/cgi/content/full/18/7/4089</p> <p>--</p>	1-20
A	<p>JBC Online, "Activation of Hypoxia-inducible TranscriptionFactor Depends Primarily upon Redox-sensitive Stabilization of Its & Subunit", Vol271. No 50, Issue of December 13, 1996, L. Eric Huang et al, http://www.jbc.org/cgi/content/full/271/50/32253</p> <p>-- -----</p>	1-20

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/12/99

International application No.

PCT/SE 99/02053

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9639426 A1	12/12/96	AU 704384 B	22/04/99
		AU 6332696 A	24/12/96
		CA 2222279 A	12/12/96
		EP 0833840 A	08/04/98
		IL 118581 D	00/00/00
		JP 11507541 T	06/07/99
		US 5882914 A	16/03/99
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